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A Study of Cellular Differentiation during Regeneration of the Lens with Specific Reference to the Mechanism of Cell Elongation during Lens Fiber Formation

by

Sharon Hornsby

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF PH.D.

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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "A Study of Cellular Differentiation during Regeneration of the Lens with Specific Reference to the Mechanism of Cell Elongation during Lens Fiber Formation" submitted by Sharon Hornsby in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

An investigation was made of the possible means of cell elongation during lens fiber formation during the process of Wolffian lens regeneration. The problem was approached from two different angles: drug disruption of specific organelles that might be involved in elongation - microtubules and microfilaments, and ultrastructural observation of cells undergoing elongation to see if organelle orientation or cell contacts could be implicated in elongation.

Results showed that regenerates treated with colchicine before or at the onset of cell elongation were inhibited from beginning or furthering cell elongation. Parallel experiments showed that cells in which cellular elongation was inhibited seemed unable to synthesize lens specific proteins, the crystallins. Ultrastructural observations showed that microtubules were absent from the cytoplasm of the colchicine treated cells. Further experiments which studied the appearance of cell surface markers at periods when colchicine inhibition was greatest showed that the appearance of these markers was also colchicine sensitive.

The suggestion was made that microtubules seem to have an important role in the elongation and differentiation

of lens fibers in the lens regenerating system. Microtubules could possibly act in cell elongation by three mechanisms: (a) forming a cytoskeleton which would support the changes in cell shape, (b) directionally transporting materials, or (c) providing a "push" at the ends of the cells. It was further hypothesized that microtubules may be involved in the appearance of cell surface groups which could affect cellular adhesiveness and thereby affect elongation.

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INTRODUCTION

Metaplasia has been defined as 'the replacement of one differentiated cell type by another' (Foulds 1969). Grobstein (1959) has pointed out that differentiation fundamentally means increase in heterogeneity - thus in this light, metaplasia means the replacement of one type of heterogeneity by a distinctively different heterogeneity. Grobstein (1959) ,has suggested that metaplasia may occur by two different methods. One method he refers to as cellular metaplasia; this entails dedifferentiation of cells followed by redifferentiation of the same cells along a different pathway; the second type of metaplasia is called tissue metaplasia, this occurs when already differentiated cells are rejected and replaced by a new type of cell generated from undifferentiated basal cells, i.e. the differentiative route of the stem cells is changed. Because of current interest in cancer research and the redifferentiation that seems to take place in cancer cells, it is the first type of metaplasia, cellular metaplasia, that has come to the forefront of current research interests.

This study deals with a system that shows cellular metaplasia in a 'controlled' sense. The term 'controlled' is used because in comparison to a cancerous system where metaplastically transformed cells do not seem to respond

to the constraints in the organism that limit growth, this system is able to exert an influence over the products of transformation. The system is Wolffian lens regeneration in the urodele *Notophtalmus viridescens viridescens*, formerly called *Triturus viridescens viridescens*. Wolffian lens regeneration involves the dedifferentiation of pigmented dorsal iris cells and the subsequent redifferentiation of the same cells into elongated lens fiber cells. In some manner not yet understood the absence of the lens stimulates the dorsal iris cells to commence division and depigmentation. After two rounds of division the cells cease dividing, start to elongate and synthesize lens specific proteins, the crystallins (Yamada 1972). It is the elongation phase with which this study is concerned. Elongation in itself is an interesting phenomena to study because it is crucial in many developmental and morphological processes. In the regenerating lens system it is even more interesting to study because the relationship of elongation with the synthesis of tissue specific proteins can be approached experimentally. Thus it would be possible to test if interference with elongation affects the synthesis of tissue specific proteins or any other cellular functions.

There are several theories that attempt to explain the mechanisms involved in changes in cell shape such as occur in cell elongation. Microtubules are often implicated in shape changes, especially those involving cell

elongation. Some investigators feel that microtubules are merely providing the skeletal frame of the cell; while others have suggested that these structures may be causal in elongation (a) by increasing in length and thereby exerting a force in the direction of elongation or, (b) by channeling cytoplasm towards the direction of elongation. (See Literature Review for a comprehensive review of this work.) Microfilaments have also been suggested as causal agents in changes in cell shape. In most of the systems in which these structures have been studied they seem to function in a different manner than microtubules. Shape changes associated with microfilaments often occur rapidly and appear to be the result of a contractile process (Cloney 1966). In some systems localized bands of microfilaments appear to act as a "purse-string" and their contraction brings about a decrease in cross-sectional area at one of the ends of individual cells (Baker and Schroeder 1967, Wessels *et al* 1971). Recent evidence indicates that some microfilaments can be decorated with heavy meromyosin; a property apparently exclusive to actin. These findings have suggested that the contractile properties associated with the microfilaments may be due to the presence of actin (Spooner *et al* 1973, Schroeder 1973, Palevitz *et al* 1974, Woolley 1970, Pollard *et al* 1970, Nachmias *et al* 1970).

From studies on amphibian neurulation, Brown et al. (1941) have concluded that differential cell adhesions are responsible for cell elongation. More recently, Benedetti et al. (1974), studying elongating lens fibers by freeze etching have suggested that certain sites visible in the freeze fractured surfaces may be responsible for reciprocal recognition of two cell surfaces or for specific cell-to-cell interlocking. Whether or not these sites have anything to do with elongation has not been discussed.

Piatigorsky *et al* (1972 a,b,c) studying developing chick lens have shown that microtubules are most likely involved in elongation. On the other hand, Wrenn and Wessels (1969) have suggested that microfilaments are involved in elongation during lens fiber formation in the mouse.

There are several ways to approach the question of what factors are responsible for fiber elongation in the lens regenerating system. The first approach is to look at the ultrastructure of the elongating cells in order to study the presence, distribution and orientation of cell organelles, in particular microtubules and microfilaments. A second approach is to use compounds that interact specifically with either microtubules or microfilaments. The effect of these compounds on fiber differentiation can then be studied. Although neither approach in itself can conclusively point out the mechanism responsible for

elongation, a combination of several methods can often lead to a more reasonable estimation of the events that are occurring at the cellular level.

There were several questions that were investigated in this study:

1. Are microtubules or microfilaments present in the lens regenerate during lens fiber formation, and if they are present what is their arrangement and orientation?
2. If either of these organelles are present are they involved in lens fiber elongation?
3. Does interference with cell elongation affect the acquisition of lens specific proteins, the crystallins? Zwann (1975) has suggested that in mutant mouse embryos where lens development ceases before elongation, no crystallins are formed.
4. Does interference with cell elongation affect any other cellular events occurring during lens regeneration, for instance the appearance of certain cell surface markers? Work with polymorphonuclear-leukocytes has shown that microtubules are possibly involved in cell surface insertion and translocation (Ukena and Berlin 1972). If fiber elongation is suppressed are these other events also affected?

In order to answer the first question I used an electron microscopic study of the ultrastructure of regenerating irises. The second question was answered by a

combination drug and ultrastructure study. Colchicine or vinblastine was used because both affect the microtubular system, while cytochalsin B was used because this compound interferes with microfilaments. Drug treatments in combination with immunofluorescence and immunoelectrophoresis studies were used to answer question 3. Drug treatments combined with cell electrophoresis determinations were used to answer question 4.

REVIEW OF THE LITERATURE

Wolffian Lens Regeneration

In 1891, Colucci reported that certain amphibians of the genus *Triturus* were able to regenerate a new lens from their dorsal iris when the original lens was removed. Subsequently Wolff (1894, 1895, 1901, 1904), using larvae and adults of *Triturus taeniatus* and *Triturus cristatus*, rediscovered this phenomenon. This interesting type of regeneration which represents cellular metaplasia, is now known as Wolffian lens regeneration. Since this early work many studies have been done on Wolffian lens regeneration, and they have been reviewed by several authors: Stone 1959, 1965, Reyer 1954, 1962, Schieb 1964, and Yamada 1967. Unlike limb and tail regeneration which is widespread throughout the order Urodela, Wolffian lens regeneration is restricted to 14 species of newts belonging to the genus *Triturus* (Schieb 1964). *Salamandra salamandra* is the only species not belonging to Triturus in which Wolffian lens regeneration has been indisputably documented (Reyer and Stone 1955).

Several properties make Wolffian lens regeneration a good system for studying the loss and acquisition of specific characteristics of cellular differentiation: 1. The eye cup is a fairly isolated environment and this

enables one to transplant tissues from one animal to another and, to a certain extent, avoid the complication of the immune response; 2. One is able to control when the initial stimulus (lens removal) is given, thus allowing the investigator to induce cellular metaplasia at will; 3. Unlike limb regeneration, where there is a great deal of controversy as to the origin of the cells forming the blastema, it is generally agreed that the cells forming the lens are derived from the iris. There are several types of experiments that have supported this assumption. Wachs (1914), Sato (1930), Mikami (1941), and Stone (1943, 1952) have all done experiments grafting pieces of dorsal iris from one newt into the lentectomized eye of another and have observed that a new lens forms from the graft. The implications of these experiments were further strengthened by grafting iris from a regenerating species into the eyecup of a non-regenerating species and observing lens formation from the implant (Ikeda 1934, Amano and Sato 1940, Reyer 1953, 1956). Tracing ^{3}H-thymidine labelled iris cells through subsequent stages of regeneration into lens cells is another method used to demonstrate that iris cells transform into lens (Eisenberg and Yamada 1965a,b, 1966, Reyer 1966, Zalik and Scott 1971).

By close histological observation of the regenerating iris in *Triturus taeniatus* and *T. pyrrogaster* (Sato 1940), *T. cristatus* (Zalokar 1944), *T. viridescens* (Reyer 1948, 1950,

Stone and Steinitz 1953a, Yamada and Roesel 1964), investigators have been able to classify the events occurring during Wolffian lens regeneration into thirteen distinct stages. Yamada (1967) using electron microscope and light microscope data as well as other 'personal observations' has further characterized Sato's stages. (See Figure 1.)

In reviewing the literature on Wolffian lens regeneration I will examine the questions that have been asked in this system and the experiments performed in attempts to answer these questions. Several basic questions can be asked about Wolffian lens regeneration. 1. What regions of the iris can produce a lens? 2. How does lens removal release the process of regeneration? 3. Can the dorsal iris regenerate when removed from the eye and isolated; if not, is there a specific factor present in the eye that allows regeneration to occur? 4. What events take place at a cellular level in the time interval between the removal of the old lens and the formation of a new lens?

1. *In vivo*, in a normal regenerating system, lens formation is restricted to the margin of the dorsal iris; however, Stone (1954a, 1954b) has been able to produce lenses from other parts of the dorsal iris (never from the ventral) by producing an artificial secondary pupil using pliofilm or by rotating this tissue 180° and grafting it so that the ciliary margin becomes part of the free

Figure 1:* Yamada's Characterization of Sato's Stages of Regeneration in *Triturus v viridescens*

Stage I (3-6 days,* Fig. 1). The interlaminar space is enlarged. In the nuclei of iris epithelial cells, the number of invaginations of the nuclear envelope is reduced. Consequently, the shape of the nuclei approaches a sphere. Surrounding the pupillary margin, free amoeboid cells are observed which, according to Eguchi (1963), participate in the process of depigmentation in the following stages.

Stage II (6-10 days, Fig. 1). Rounding-up of nuclei of iris epithelial cells continues. The pigment granules in the cells at the mid-dorsal margin are being reduced in number. A number of amoeboid cells begin to appear in the expanding interlaminar space, as well as surrounding the iris epithelium. Eguchi (1963) suggests that pigment granules of iris cells are removed by these amoeboid cells through their prominent pseudopodia.

Stage III (8-11 days, Fig. 1). Depigmentation of cells at the dorsal pupillary margin continues, giving rise to a group of almost completely depigmented cells in the internal lamina and at the very margin of the mid-dorsal iris. Within the depigmented or depigmenting cells the number of free ribosomes is increasing, there is more rough-surfaced endoplasmic reticulum, and the ultrastructure of the nucleoli is becoming more elaborate (Karasaki, 1964; Eguchi, 1964). Many of the amoeboid cells are heavily loaded with pigment granules.

Stage IV (9-15 days, Fig. 1). The layer of depigmented cells forms a vesicle (lens vesicle) at the mid-dorsal pupillary margin without losing its connection with the external and internal laminae of iris epithelium. The cavity of the vesicle is continuous with the interlaminar space of the iris. Often a small number of cells, apparently coming from the internal lamina of iris epithelium, is observed within the vesicle cavity in *T. viridescens.*

Stage V (12-15 days, Fig. 1). The lens vesicle is enlarged by the addition of depigmented cells as well as by rapid cell multiplication of depigmented cells. The internal layer (lower in figure) of the vesicle is thickening. In some regenerates the proximal part (left in figure) of the internal layer becomes irregular, apparently owing to accumulation of a large number of cells. Although the external and internal layers of the lens vesicle are continuous with the external and internal laminae of iris epithelium, the boundary between the depigmented and pigmented areas is often indicated by a weak constriction. The cytoplasm of all vesicle cells shows a high density of free ribosomes and heavy basophilia sensitive to ribonuclease.

Stage VI (12-16 days, Fig. 1). The central part of the internal layer has thickened by elongation of individual cells. At this stage these cells, which are going to form the primary lens fibers, lose the cytoplasmic basophilia which was strong in stage IV. Mitoses are not observed in this thickened part, but are frequent in all other depigmented areas. From the lens vesicle proper, a short stalk-like portion is constricted, which is directly connected with the pigmented area. The stalk is composed of external and internal layers. Apparently, cells are flowing continuously from the pigmented area into the vesicle through the stalk.

Stage VII (15-18 days, Fig. 1). A further thickening of the main part of the internal layer has led to a protrusion of its inner surface into the cavity of the vesicle (the fiber hillock). All cells in this thickened area are elongated and irregularly arranged in approximately two cell layers. Most of the surface area of such primary fiber cells is in contact with surfaces of the neighboring primary fiber cells, reducing the free surface to a minimum. The primary fiber cells begin to show acidophilia in their cytoplasm, after completely losing the cytoplasmic basophilia of the preceding stages. By contrast, the cells in the external layer of the vesicle and those in the stalk retain strong cytoplasmic basophilia.

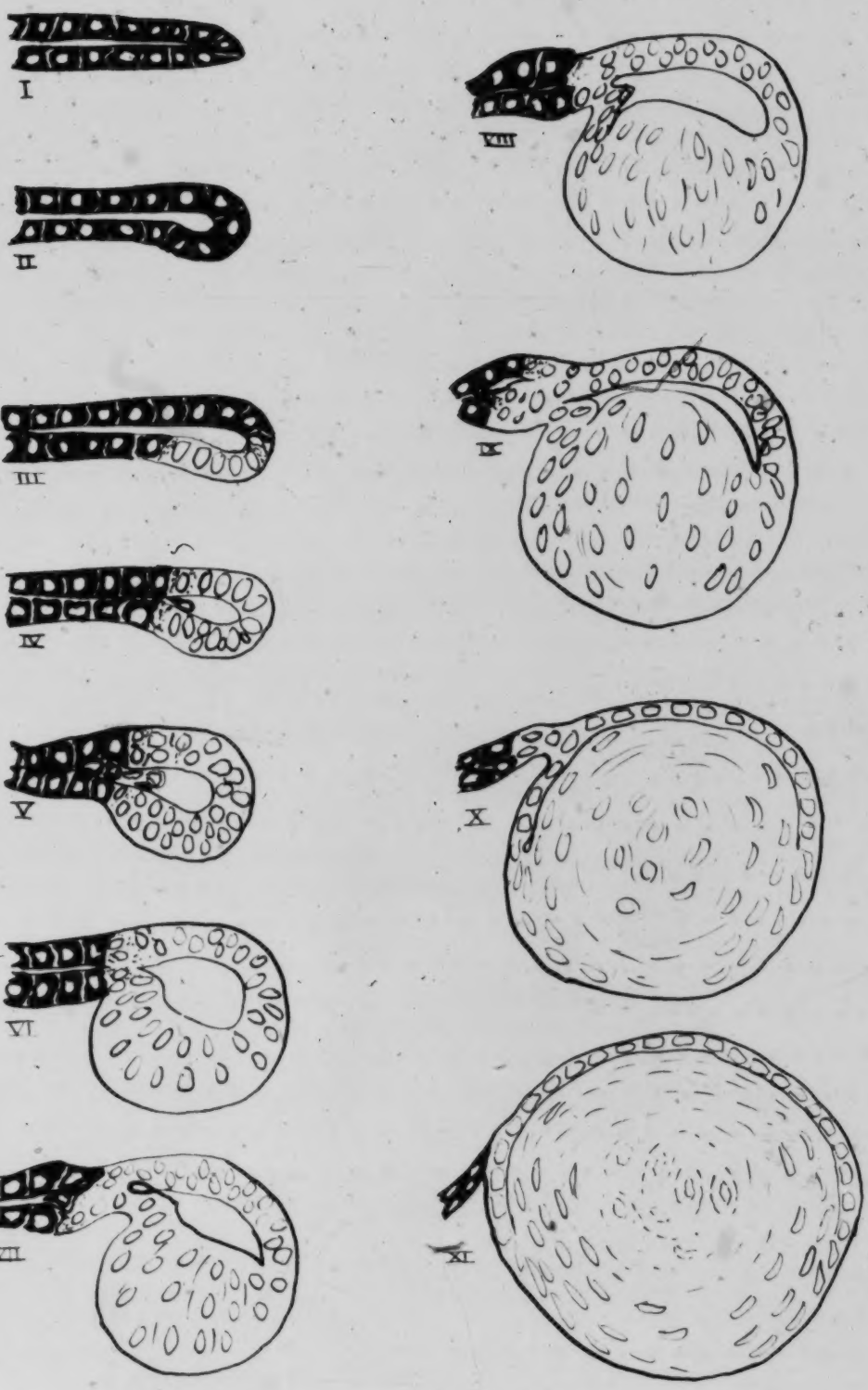
Stage VIII (15-19 days, Fig. 1). Further expansion of the fiber cell mass reduced the central cavity of the lens vesicle to a narrow slit. Between the elongated primary fiber cells and depigmented cuboidal cells there are a number of cells in the beginning phase of elongation; these are the first generation of the secondary fiber cells.

Stage IX (18-20 days, Fig. 1). The whole external layer and the proximal part of the internal layer become flattened and start to encircle the fiber cell body, which is increasing in size through growth of individual fiber cells and addition of new fiber cells from the periphery. Frequent mitotic figures are observed in the whole external layer, in the proximal part of the internal layer, and in the stalk region. Elongation of the secondary fiber cells is occurring rapidly at the equatorial zone. The cytoplasmic acidophilia of these cells is increasing, but it is stronger in the primary fiber cells that constitute the core (nucleus of the lens body).

Stage X (18-25 days, Fig. 1). In the whole external layer and in the proximal part of the internal layer, the cells are arranged more regularly in one cell layer. Now these layers can collectively be called the lens epithelium. The lens epithelium is applied to the fiber cell complex and is still connected through a stalk with the dorsal iris epithelium. Elongating secondary fiber cells completely embrace the core of primary fiber cells. In the primary fiber cells, the nuclei are becoming less basophilic and increasingly acidophilic.

Stage X (21-28 days, Fig. 1). The lens is growing rapidly due to growth of secondary fiber cells and proliferation in the lens epithelium. The cells of the first generation of secondary fibers are elongated and form a thick concentric layer surrounding the primary fiber core. The stalk disappears, and the new lens becomes an independent tissue. Due to loss of basophilia and acquisition of acidophilia, the nuclei of the primary fibers are very difficult to distinguish from the cytoplasm.

*These drawing and descriptions were taken from Yamada (1967).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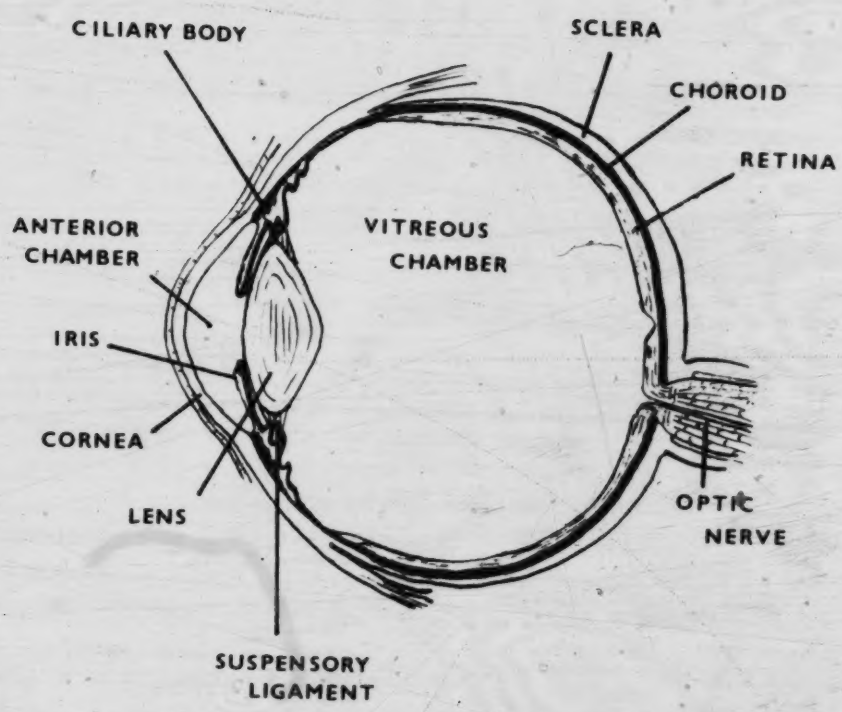
pupillary border. (See Figure 2 for a labelled diagram of the eye.) Using these methods he has also been able to map the capacity for lens regeneration in various regions of the dorsal iris. He has found that the potency for lens regeneration "decreases dorsally towards the ora serrata and disappears in the nasal and temporal regions slightly above a line passing nasotemporally through the midpupillary space" (Stone 1954a). Recently Eguchi & Watanabe (1973) have been able to stimulate the ventral iris to form lentoids by using chemical carcinogens.

2. There are five theories as to how the presence of a normal lens can block regeneration (Reyer 1962): 1. the lens exerts pressure on the iris and thereby inhibits its activation; 2. the lens and the suspensory ligament act as a barrier which prevent the diffusion of a stimulating retinal factor from the neural retina to the iris; 3. the metabolism of the lens uses up the retinal factor which is then no longer available to activate the iris; 4. the lens secretes some substance which neutralizes the action of the retinal factor; 5. the lens secretes some factor which inhibits regeneration of another lens from the iris. Experimental evidence seems to point to a combination of theories 2, 3, and 4 or 5. Reyer (1961) showed that iris implants into the vitreous chamber with the host lens in position were able to form a lens; but, similar implants into the anterior chamber did not regenerate. This

Figure 2:* A Labelled Diagram of the Eye

*This diagram was adapted from several diagrams in Davson (1962)


CILIARY BODY
SCLERA
CHOROID
RETINA
ANTERIOR CHAMBER
VITREOUS CHAMBER
IRIS
CORNEA
LENS
OPTIC NERVE
SUSPENSORY LIGAMENT

suggests that the lens is acting as a barrier for a diffusible factor from the neural retina. Zalokar (1944)was able to inhibit lens regeneration by implanting kaolin into lentectomized eyes. Since kaolin was believed to act by absorbing the neural retinal secretion it was suggested that the lens may function in a similar manner by actively metabolizing its neural retinal secretion. Stone and Vultee (1949), using *T. viridescens*, injected aqueous humor from an eye containing a lens into a lentectomized eye. Lens regeneration was inhibited for up to three months. This experiment suggested the presence of some inhibitory factor (theory 4 or 5) secreted into the aqueous humor. However, Takano *et al* (1957), using *T. pyrroghaster*, were not able to verify these results. In summary it appears that removal of the lens may release regeneration in that: a) the barrier keeping the retinal factor from reaching the iris is removed, b) the tissue which metabolizes the retinal factor is gone, c) the factor secreted by the lens to inhibit the retinal factor or to inhibit regeneration is no longer present.

3. Using an *in vivo* system, Stone (1958) has shown that regeneration can be inhibited or halted by isolating the iris from the neural retina. Stone and Steinitz (1953a) have shown that if the neural retina is removed at the time of lentectomy, lens regeneration is inhibited until the neural retina has begun to regenerate. Reyer and

Withersty (1972) and Reyer *et al* (1973) were able to obtain lens regeneration from dorsal iris when it was implanted into the blastema of a regenerating limb, but not if the blastema was denervated. These observations imply that a neural factor is probably essential to stimulate regeneration. Attempts were made to study lens regeneration in hypophysectomized animals. Stone and Steinitz (1953b) and Connelly *et al* (1973) found that hypophysectomy retards lens regeneration but does not completely inhibit it.

Early attempts to obtain lens regeneration from the dorsal iris alone *in vitro* (Zalokar 1944, Stone and Steinitz 1958, Eguchi 1967, Eisenberg-Zalik and Meza 1968, and Zalik and Scott 1969) were unsuccessful at obtaining final stages of lens regeneration from the dorsal iris. Eguchi (1967) was able to get lens regeneration if he included a piece of neural retina from lentectomized or non-lentectomized eyes. Yamada *et al* (1973) have been able to get lens formation *in vitro* by including a piece of retina from frog larvae. Recent observations by Horstmann and Zalik (1974) and Eguchi *et al* (1974) indicate that cultures of iris cells are able to form lentoid-like areas even when no retinal factor is supplied. Moreover Eguchi *et al* (1974), using fluorescent antibodies, have observed lentoid-like areas which stain with antibodies against lens crystallins in cultures of ventral iris.

4. The cellular events occurring during lens regeneration have been studied by a number of investigators. A series of changes takes place in the nucleus and cytoplasm of the iris cells as they are converted into lens cells. Eisenberg and Yamada (1965a and b) and Reyer (1966) have analyzed DNA synthesis in *T. viridescens* using autoradiographic techniques. They have found that DNA synthesis is activated around 5 days after lentectomy in both the dorsal and ventral iris. Incorporation of ^{3}H-thymidine into cells, a measure of DNA synthesis, continues, until stage IV (9 - 15 days) when a small group of cells ceases to synthesize DNA. The number of these cells increases with time and after Stage VI (12 - 16 days) they begin to differentiate into primary lens fibers. Yamada and Roesel (1971) have shown that there are two peaks of mitosis occurring during regeneration - one at 7 days and one at 15 days post-lentectomy.

The earliest sign of regeneration is the onset of nuclear RNA synthesis, detected one day after lentectomy (Yamada and Karasaki 1963, Reese *et al* 1969). Ribosomal RNA synthesis increases between 2 - 10 days after lentectomy. At the same time there is an observable alteration in nucleolar ultrastructure indicating nucleolar activation (Karasaki 1964, Dumont *et al* 1970). The cell then enters into the cell cycle (Eisenberg and Yamada 1965a and b), Eguchi 1964). As these changes are taking place the

cytoplasmic and nuclear volume of the pigmented iris cell increase until 6 - 8 days post lentectomy, then as differentiation occurs the cytoplasmic volume is greatly reduced while the nuclear volume remains constant (Dumont and Yamada 1972). The reduction in cytoplasmic volume has been called cytoplasmic shedding and Yamada (1971) has shown that incorporation of ^{3}H cytidine into the nuclear RNA fraction is greatly enhanced after cytoplasmic shedding is completed. He suggests that:

> the cytoplasm contains a number of factors which exert specific repressive controls over nuclear synthetic activities and that cytoplasmic shedding removes such repression so that the cell can be reprogrammed in a new direction.

As cytoplasmic shedding and depigmentation of the iris cells occurs, macrophages, which have invaded the iris, remove the cytoplasmic debris (Eguchi 1963, Karasaki 1964, Dumont and Yamada 1972, Yamada and Dumont 1972).

Autoradiographic studies have shown that there is an increase in protein synthesis in the activated dorsal iris. This phenomenon occurs in two phases; in the first one which occurs between 2 and 8 days after lentectomy, activation of protein synthesis, can be detected in all cells of the dorsal iris. In the second phase, which occurs 16 to 25 days after lens removal, enhancement of protein synthesis is more pronounced and is localized in the elongating lens fiber cells. Immunofluorescence studies (Ogawa 1963, Takata *et al* 1964) using antiserum

against total lens showed that a weak fluorescence is already present in a few cells of the regenerate by Stage IV (9 - 15 days). Using antiserum specific for gamma crystallins Takata *et al* (1965, 1966) were able to detect gamma crystallins at Stage V (12 - 15 days). Yamada and Takata (1965) combined an autoradiographic and immunofluorescence study to show that no cell synthesizes detectable gamma crystallin until one day after it has passed through its final DNA synthesis period.

Studies on the cell surface using cell electrophoresis have also been done on regenerating iris cells (Zalik and Scott 1972, 1973). These studies have shown that there is a significant decrease in the cell surface charge between 10 and 15 days after lentectomy and that dedifferentiating cells lose neuraminidase and RNase sensitive groups at the cell surface. The authors have suggested that the loss of these groups may be an event in dedifferentiation that enables the cells to change their differentiative route.

> One of the first signs of differentiation in many cells is the development of asymmetric cell form. Examples would include the development of a cilium or a flagellum, or the elongation of a cell or part of a cell such as occurs in the development of a muscle cell, nerve cell or lens cell. (Tilney 1971)

Extending this hypothesis to the Wolffian lens regeneration system, it would appear that the elongation of the depigmented iris cells is one of the first signs of

differentiation into lens fiber cells. The first primary lens fiber cells start to elongate in late Stage IV (9 - 15 days) or early Stage V (12 - 15 days) and it is at these stages that the first lens specific crystallins have been detected. Elongation in these cells is important for several reasons: 1) it appears to be necessary for the formation of the lens fiber cells and the synthesis of crystallins (Zwann 1975); 2) it is an indication of the onset of redifferentiation. It would therefore be valuable to know how these cells elongate.

Microtubules

Microtubules are the cell organelle most often cited as major contributors to cell elongation. Early workers regularly observed microtubules in the mitotic apparatus (Mazia 1961), dendrites of neurons (Palay 1956), cilia and flagella (Roth 1957, 1958), sperm tails (Burgos and Fawcett 1955), axostyles (Grassé 1956) and even in the cytoplasm of some cells (Slautterback 1963). Along with the introduction of glutaraldehyde as a general purpose fixative (Sabatini *et al.* 1963) came the realization that microtubules were ubiquitous cell organelles. Since 1963 microtubules have been implicated in many systems as being the causal or at least the supportive agents in cell elongation. Wessels (1971) has described how microtubules act as a skeleton in many systems from neuronal

axons to migrating cells from the embryonic heart. Burnside (1971) has suggested that in newt neurulation microtubules probably contribute to cell elongation by some form of cytoplasmic transport. In *Xenopus* neurulation, Karfunkel (1971) suggests that microtubules are probably only responsible for the maintenance of cell shape. Handel and Roth (1971) also concluded that microtubules contribute to the maintenance of cell shape in the chick neural tube. Daniels (1972) has shown that inhibition of microtubule assembly halts the formation of nerve fibers. Tilney and Porter (1967), Tilney (1968), Kitching (1964), Tilney *et al.* (1966) and Roth and Shigenaka (1970) have all shown that microtubules are responsible for the extension of the axoneme of the long rigid axopodia of the heliozoan *Echinoshaerium nucleofilum*. Piatigorsky has reported that "*de novo* synthesis of protein, possibly microtubular protein" is responsible for continued elongation of chick lens epithelial cells. Kuwabara (1968) has found microtubules in adult human lens cells and has suggested that they play a role in maintaining cell shape and rigidity. A study of the developing lens of squid has suggested to Arnold (1966) that "the presence of microtubules may be correlated with elongation, the movement of materials into the lens region or possibly with the secretion of lens material". Byers and Porter (1964) have observed the presence of microtubules in the

invaginating chick lens placode. Working in the same system Pearce and Zwann (1970) have concluded that microtubules are not necessary for the maintenance of cell shape once the elongate shape is reached. They came to this conclusion after treating the cells with colcemid and observing that although the treatment caused the disappearance of microtubules it did not in any manner alter the shape of the previously elongated cells. This observation, however does not eliminate microtubules as causal factors in initial cell elongation .

In addition to their suggested role in cell elongation, microtubules which have been found in other systems have been assigned many diverse roles from that of 'railroad tracks' for delivering products,to involvement in membrane insertion. Hepler and Newcombe (1964) found that microtubules are somehow involved in determining the place of deposition and the orientation of the cell wall microfibrils in cells of *Coleus* undergoing secondary wall deposition. Goldman (1971) has suggested that microtubules play important roles in the determination of cell shape, the formation of major cell processes, and in cell locomotion of BHK 21 fibroblast-like cells. Kessel (1966) studying spermiogenesis in the dragonfly has implied that the orientation of groups of microtubules in association with the nucleus are responsible for elongation and pattern formation in the nucleus. MacGregor and Stebbings

(1970) have postulated an interesting role for microtubules in the telotrophic ovarioles of the 'backswimmer' *Notonecta*. They suggest that microtubules are responsible for facilitating and directing the flow of ribosomes from trophocytes to oocytes via the trophic core and trophic tube by keeping these regions open. Hasskarl *et al* (1973) have also postulated a guidance role for microtubules in tracheole migration in wing discs of *Galleria mellonella*. They have shown that microtubules are necessary for the tracheoles to migrate into the lacunae of the wing imaginal discs in culture. The plentiful occurrence, orientation and grouping of microtubules and microfilaments during scale morphogenesis in *Ephesta Kichniella* has suggested to Overton (1966) that the microtubules and microfilaments have an important role in that system as well. Observations by Moran and Varela (1971) have implicated microtubules in sensory transduction in campaniform sensilla (mechanoreceptors associated with the cuticle) in cockroach legs. Sanders and Zalik (1970) have shown that microtubules are present during the separation of hypoblast cells from epiblast cells in pre-streak chick blastoderm. Granholm and Baker (1970) have related the presence of microtubules in the primitive streak to cell invagination leading to the formation of the mesoblast in the chick blastoderm. Goodman *et al* (1970) have shown a possible relationship between cyclic AMP and neurotubules (the name

for microtubules in the nervous system) in synaptic transmission. There is evidence suggesting that microtubules may also be involved in membrane insertion and organization. Results of other investigators also suggest that microtubules may have a role in controlling the distribution of adhesive components in cell surface and in the stabilization of antigenic determinants and other functional elements in hamster fibroblasts, (Waddell, Robson, and Edwards 1974). Others working with polymorphonuclear leukocytes have shown that microtubular integrity is essential for topographical reorganization of the cell surface after phagocytosis (Oliver, Ukena and Berlin 1974; Ukena and Berlin 1972).

Microtubules seem to have many diverse yet important roles therefore it is important to review what is known about them biochemically and structurally. The structure of the microtubule and its subunits has been investigated by several workers. The microtubule itself has a diameter of 225-250 A with a central core of 150 Å. In cross-sections a microtubule appears as a cylinder whose wall is composed of 13-15 globular subunits. The subunits stack linearly and in longitudinal section the microtubule appears as if it is forming a beaded filament (Kiefer *et al* 1966). The protein composing the subunit is called tubulin and various estimates of its weight have been made. Early estimates by Kiefer suggested a sedimentation

coefficient of about 2.5S whereas Shelanski and Taylor (1967) have suggested 6S. Weisenberg *et al* (1968) have found that tubulin normally exists as a dimer MW 120,000 S^0_{20W} - 5.8S which in 6M guanidine hydrochloride is converted to the monomer-MW 60,000. Recent evidence seems to indicate that there are two types of tubulin, called either tubulin A and B or tubulin 1 and 2; these categories were established when it was found that tubulin migrated as two bands during gel electrophoresis. (Bibring and Baxandall 1971, Stephens 1970). It is still not known if tubulin exists as a homodimer or heterodimer in its native state.

Microtubules are usually straight and may be several microns long; some investigators have noticed bridges between microtubules. Tilney (1971) has suggested that bridging of microtubules may be important in the formation of the axoneme in *Radiophrys*. McIntosh (1974) has observed bridging in the axostyle of *Saccinobaccilus* and in the helix of tubules surrounding elongating spermatid nucleus and the flagellum of the sperm tail in chicken testis. Bhisey and Freed (1971) have found cross-bridges on the microtubules of cooled interphase HeLa cells. Friedman (1971) has reported arm-bearing microtubules associated with an unusual desmosome-like junction in adult cricket tibias. Besides many other observations like the above, Behnke and Forer (1972) working on cranefly testis have shown that

microtubules can be transformed into a helical structure by vinblastine treatment. These observations seem to indicate that microtubules can have arms and that under different circumstances microtubule protein can pack into polymeric forms different from that of the microtubule.

Early work has suggested that there may be similarities between actin and tubulin. Mohri (1968) and Oosawa *et al* (1966) reported that there were similarities in amino acid sequence between actin and tubulin from different sources. Similar molecular weights and electrophoretic mobilities have also been demonstrated by several investigators (Renaud *et al* 1968, Stephens (1968a)). However Stephens and Linck (1969) have shown that tubulin and actin from the same organism, *Pecten irradians*, had very few amino acid residues in common. Stephens (1968b) has also shown that although actin and tubulin bind a nucleotide, actin binds ADP, whereas neurotubular subunits bind GDP. Weisenberg *et al* (1968) have shown that the tubulin dimer binds two moles of GDP - one reversibly and one irreversibly. Olmsted and Borisy (1973) have suggested that actin and tubulin have possibly evolved from a common ancestral protein.

Several metabolites can affect microtubular function and structure. Cyclic AMP seems to affect functions which require intact microtubules. Ortiz *et al* (1973) working on cultured iris epithelial cells have shown that

cAMP can cause shape changes in the cells possibly by affecting microtubular formation. Wolfe (1973) studying *Tetrahymena* has suggested that cAMP stabilizes microtubules in the same manner as D_2O. Creighton and Trevithick (1974) studying cultured epithelial cells of 1 day old rat lens found that cAMP stimulated cell growth and elongation and accelerated the appearance of gamma crystallins. Gillespie (1971) working on rat spleen and liver has suggested that Ca may also be involved in microtubular assembly and she and others have summed up the relationship as follows:

low cAMP, Ca^{++} bound or outside

↓

subunits $\overset{Ca^{++}}{\underset{\text{high-cAMP}}{\rightleftarrows}}$ Microtubules

According to her scheme cAMP can either stimulate or inhibit microtubule association depending on its concentration. These results are interesting since Weisenberg (1972) has shown that Ca^{++} inhibits microtubular polymerization *in vitro*.

Microtubules have been shown to be unstable under conditions of cold (Tilney and Porter 1967), colchicine (Tilney 1968), cupric and nickelous ions (Roth and Shigenaka 1970), and vinblastine (Marantz *et al* 1969) - contrasted to this is their marked stability in the presence of D_2O (Gross and Spindel 1960, Inoué and Bajer 1961) and hexylene glycol *in vitro* (Kirkpatrick 1969). Behnke and

Forer (1967) have shown in cranefly spermatids, rat sperm and rat tracheal cilia that microtubules can be classified into four categories according to their reactions to varying temperature, colchicine, pepsin treatment and negative staining at pH 7: cytoplasmic microtubules, accessory tubules (of 9 + 2 complex), B tubules (of the 9 + 2 complex), and A tubules (of the 9 + 2 complex) (rat sperm and rat tracheal cilia contain only 9 + 2 tubules). Colchicine treatment or 0°C causes the disappearance of cytoplasmic microtubules. At 50°C cytoplasmic microtubules are the first to disappear followed by B tubules and finally by A tubules. Brief pepsin treatment causes the 9 doublets to disappear. When negative staining at pH 7 is used no cytoplasmic microtubules are present, central microtubules are infrequently observed , B tubules are fragmented and A tubules are intact.

One of the problems with studying microtubules is determining exactly when they arise and their source within the cell. Besides their association with centrioles during mitosis, and basal bodies in cilia and flagella, microtubules have been associated with centriolar satellites (deThé 1964, Robbins *et al* 1968), bodies free in the cytoplasm (Bowers and Korn 1968), membrane associated bodies (Satir and Stuart 1965), portions of the nuclear envelope as in dividing yeast cells (Robinow and Marak 1966) the kinetochore (Brinkley and Nicklas 1968), the diffuse

density seen at the midbody in animal cells (Robins and Gonatas 1964), and in the phragmoplast of plant cells (Hepler and Jackson 1968). This type of observation has led some investigators (Porter 1966, Inoué and Sato 1967, Tilney 1968, Gibbons *et al* 1969) to suggest that the above mentioned structures may control microtubule distribution by acting as a nucleating centre. Pickett-Heaps (1969) has suggested that microtubule organizing centres and not centrioles are responsible for the organization of the mitotic apparatus. He suggests that the centrioles are only responsible for flagella and cilia and that they just "go along for the ride" during mitosis. Went (1959) showed immunochemically that sea urchin eggs contain a precursor for the mitotic apparatus, therefore tubulin does not have to be synthesized *de novo* before it is organized; this idea has also been suggested by Piatigorsky *et al* (1972b) in developing chick lens. Burgess and Northcote (1968) suggest from observations on mitosis in dividing plant cells (wheat root tips) that endoplasmic reticulum is responsible for transport of tubulin to sites of microtubular aggregation and disaggregation. A method of observing microtubular initiation has been developed by Spendlove using mammalian cells (Spendlove *et al* 1964, Spendlove *et al* 1963 a and b, Spendlove 1970, Kohler and Spendlove 1974). They use reovirus which coats microtubules and tubulin in mammalian systems. Fluorescent

antibody against reovirus was used to show whether or not microtubular subunits are polymerized or unpolymerized.

If microtubules are responsible for elongation how do they accomplish this process? It has already been suggested that they do so by some sort of cytoplasmic transport (Burnside 1971, Arnold 1966). Inoué (1959) and Inoué and Sato (1967) have suggested that chromosome movement and cell shape changes are based on labile association of molecules controlled by organizing centres and other factors. Stephens (1973) doing a thermodynamic analysis of dividing sea urchin eggs at different temperatures has shown that Inoué's postulate of first order polymerization and dissociation kinetics is the best way to describe mitotic spindle motions.

Microfilaments

Another cell component which has been implicated in cell shape changes is the microfilament. Since the integrity of microfilaments is not dependent on glutaraldehyde fixation as are microtubules, these structures have been observed since the advent of the electron microscope. In spite of this, very little work has been done on them until recently. McNutt *et al* (1971) have classified the microfilaments present in 3T3 cells into two categories: 1. alpha filaments, 60-80 Å . These filaments which appear as felt-like meshworks attach to the cytoplasmic

surface of the plasma membrane; 2. Beta filaments, 100 Å , which run in loose fascicles, frequently surround organelles such as the smooth endoplasmic reticulum and are particularly prominent near the Golgi complex. The authors have suggested that the alpha filaments resemble 70Å filaments which are believed to have contractile properties in a number of cell types. Ishikawa *et al* (1969) have shown that heavy meromysin binds to actin filaments in the cytoplasm of many cells. These actin filaments are now thought to be the same as the microfilaments 50-70Å in diameter (Pollard and Weihing 1974).

The development of the drug cytochalasin which disrupts the organization of microfilaments has greatly aided the study of these structures. Most studies using this drug have shown that microfilaments play a role in cell contractility: as contractile elements involved in cytoplasmic streaming (Kamiya 1960), in inducing changes in cell shape which occur during invagination or evagination in embryogenesis, (Wrenn and Wessells 1970, Spooner and Wessells 1970), in cell locomotion (Spooner *et al* 1971), in neurulation (Schroeder 1970, 1973, Karfunkel 1971). Wrenn & Wessells (1969) without using cytochalasin B have postulated a contractile role for microfilaments during lens invagination in the mouse. Cytochalasin B has also been shown to affect the uptake of glucose (Cohn *et al* 1972). It was suggested that this was primarily by an

action on the cell membrane. Studies have also been made on the effect of cytochalasin B on actin. Cytochalasin B has been shown to induce the collapse of F-actin filaments (30-70 A) and to decrease the intrinsic viscosity of F - actin (Spudich & Lin 1972). In these studies it is important to note that cytochalasin B does not affect microtubules and that colchicine does not affect microfilaments, therefore the respective roles of each of these organelles in cell function can be elucidated in systems where both of these structures are present. Not all workers accept the hypothesis that cytochalasin B acts as a disruptive agent on microfilaments. Estensen, Rosenberg and Sheridan (1971) are just one group that have pointed out that the evidence for unequivocally accepting the relationship between microfilaments, contractile processes and cytochalasin B is open to criticism in several areas.

Some workers have indicated that their findings suggest that microtubules and microfilaments work together in some form of co-operation. This is not a new idea since many authors, mentioned previously in this review (Burnside 1971, Karfunkel 1971 and Handel & Roth 1971) already mentioned have discussed how an intact framework of microtubules is necessary before the contractile microfilaments can function. Yamada *et al* (1971) have been able to show a relationship between microtubules and microfilaments in nerve axon growth. They have shown by electron microscopy

that 40-60 A filaments have one end inserted in the plasma membrane and the other on the walls of the microtubules.

MATERIALS AND METHODS

A. Lentectomy and Iridectomy of Newts

Adult newts, *Notopthalmus viridescens*, formerly *Triturus viridescens)* were supplied by Mr. Glen Gentry and Mr. Mike Tolley, Tennessee. They were stored at 8°C in aereated tanks. Prior to the desired operations the newts were anaethesized with MS 222 (Tricaine Methanesulfonate-Sandoz Pharmaceuticals, Hanover, N.J.) and were lentectomized after the method of Eisenberg and Yamada (1966). A horizontal incision was made through the cornea and the lens was extracted by exerting gentle pressure on the cornea. Following the operation the newts were kept at 22.5°C in plastic containers filled with dechlorinated tap water. The newts were routinely fed once a week on *Enchytraeus* worms.

After appropriate intervals the dorsal irises of the newts were removed according to the method of Eisenberg-Zalik and Scott (1969). The newts were placed for 2 hours in dechlorinated tap water containing several crystals of potassium permanganate and then anaethesized in MS 222. The following procedures were carried out in a sterilized culture hood. The original corneal incision was reopened with a sharp scalpel blade and extended to the lateral and median extremes of the eye. With the help of iris scissors

the incision was then extended upward and circumferentially along the upper margin of the eye until it joined the initial corneal incision. The iris and attached cornea were placed in 0.118 M PO_4 buffer pH 7.4. The cornea and any adhering vitreous material were then removed from the iris with two pairs of watchmaker forceps. The iris was trimmed with iridectomy scissors so that only the central region containing the regenerate remained. The iris was then ready to be cultured under appropriate conditions.

B. Culture Conditions

All experiments were performed at 22.5°C. The trimmed irises were cultured for 24 hours in 50% v/v L-15 (Leibovitz) medium (Grand Island Biological Company) diluted with sterilized distilled water and containing 50 IU/ml Penicillin-Streptomycin (Grand Island Biological Company). This medium will be referred to as the control medium. In experiments designed to investigate the effect of certain drugs on regeneration, colchicine (Sigma Chemical Company), vinblastine sulfate (Sigma) or Cytochalasin B (ICI Laboratories, Chesire, England) were dissolved in the culture medium. Lumicolchicine was prepared by ultraviolet irradiation of colchicine according to the method described by Wilson & Friedkin (1966). There were usually 10-20 irises per 5 mls of medium in a 60 x 15 mm sterile plastic petri dish (Falcon Plastics). After 24 hours

incubation at room temperature, the irises were briefly rinsed in control medium and were then implanted into the eye of a freshly lentectomized newt, one iris implanted per eye. The host newts were then placed in plastic containers containing moist tissue paper for 5 days. After this time the tissue paper was removed and the containers were filled with dechlorinated tap water. This incubation of the treated iris in a host lentectomized newt is hereafter referred to as *in vivo* incubation. At 0-25 days after iris implantation, the host newts were anaesthesized, sacrificed and either the iris implant or the whole eye was processed for either light microscopy, fluorescent antibody staining or electron microscopy.

1. Operation Control

Experiments were done in order to determine the effects of *in vitro* and *in vivo* culture conditions on the control implanted irises. Irises at 0, 2, 5, 6, 7, 10, 15, and 20 days after lentectomy, were cultured for 24 hours *in vitro* in control medium and then were cultured a further 15 days *in vivo* in the eye of a lentectomized newt. They were subsequently fixed and processed for light microscopy observations.

2. Dose Response

In order to determine the non-lethal yet effective dosages of drugs on the cultured and subsequently implanted

iris, studies were done using variable drug concentrations of colchicine and vinblastine. The effects were tested over a range of doses reported in the literature to have an effect on microtubules:

Colchicine: 0.005 mg/5 ml medium - 2.5×10^{-6} M
0.05 mg/5 ml medium - 2.5×10^{-5} M
0.5 mg/5 ml medium - 2.5×10^{-4} M
2.5 mg/5 ml medium - 1.25×10^{-3} M

Colchicine was used under dark conditions as much as possible in order to prevent its conversion to lumicolchicine The correct amount of colchicine was weighed in a petri dish to which 5 mls of control medium were subsequently added.

Vinblastine: 0.02 mg/5 ml medium - 4.4×10^{-6} M
0.002 mg/5 ml medium - 4.4×10^{-7} M
0.0002 mg/5 ml medium - 4.4×10^{-8} M

A stock solution of vinblastine was prepared by adding 10 mls of sterile distilled water to a sealed vial containing 10 mg of vinblastine.

3. Most Effective *in vivo* Incubation Time

The experiments were designed in order to determine how long the irises had to be cultured *in vivo* before the drug effect was apparent and for how long the effect remained apparent. For this study 15 day regenerate irises were cultured for 24 hours either in control medium or in medium containing 2.5×10^{-5} M colchicine. Control and

drug treated irises were then implanted into the host newt. At varying times after iris implantation, host newts were sacrificed, eyes were fixed and processed for histological observation. Host animals were sacrificed at 5, 10, 15, 20, and 25 days after treated irises had been implanted.

4. Most Sensitive Stage

In order to determine the sensitivity of different regeneration stages to a particular drug treatment, irises at different regenerative stages were cultured in the presence of the drugs mentioned previously. Since it is not possible to accurately stage the regenerates without histological examination of them the period between lentectomy and removal of the irises, in days, was used as a reference for this experiment, i.e. 15 day regenerates were removed from newts 15 days after lentectomy. In one series of experiments, irises were removed 0, 2, 5, 6, 7, 10, 15, and 20 days after lentectomy, were cultured for 24 hours in medium containing 2.5×10^{-5} M colchicine/5 ml medium, or control medium, and then cultured a further 15 days *in vivo*. The hosts were then sacrificed and the eyeballs containing the implant were prepared for light microscopy. An additional experiment in this series was performed, in which seven day irises were left in the host for 20 days. In other series of experiments, irises were removed 0, 2, 6, 10, and 15 days postlentectomy and were cultured for 24 hours in control medium or in medium

containing one of the following compounds: a) 5 µg/ml cytochalasin B, b) 5 µl/ml Dimethylsulfoxide (DMSO), a solvent for cytochalasin B , c) 4.4×10^{-7} M Vinblastine .

5. Lumicolchicine

In order to determine whether or not colchicine was affecting cellular processes not involving microtubular assembly, lumicolchicine was tested on the system. Lumicolchicine was prepared according to the method of Wilson & Friedkin (1966) - colchicine was dissolved in 95% ethanol and then irradiated with ultraviolet light for 24 hours. The conversion to lumicolchicine was judged to be complete when the absorbance of the solution changed from 350 mµ to 305 mµ. Lumicolchicine was recovered from the solution by evaporation of 95% of the solvent. Colchicine treated in this manner no longer exhibits anti-mitotic or microtubular disrupting properties. Fifteen day regenerate irises were incubated for 24 hours *in vitro* in medium containing 7.5 mg/5 ml medium lumicolchicine and then for 15 days in a host newt.

D. Specimen Preparation

1. Light Microscopy Studies

The newts were decapitated and the heads were placed in Bouins' fixative. After a minimum of one day in the fixative the heads were placed in 70% alcohol for 24 hours. Using a sharp scalpel and fine scissors the eyecups

containing the implanted irises were removed from the heads and placed in fresh 70% alcohol. The tissues were then dehydrated, placed in benzene and embedded in paraplast (Fisher Scientific). The blocks were then sectioned on a Spencer 820 microtome at 7 microns; sections were placed on glass slides and stained with Ehrlich's hematoxylin-eosin. Initial observations were done blind .

2. Electron Microscopy Examination

Fifteen day regenerate irises cultured for 24 hours in control medium were removed from the culture medium and immediately fixed in 3% glutaraldehyde (LADD Research Industries) in 0.1M PO_4 buffer pH 7.4 for 3 hours at room temperature. The following schedule for electron microscopy fixation was then followed:

rinse in .1M PO_4 buffer pH 7.4 for 5 minutes and then in fresh buffer overnight

postfix in 2% OsO_4 (LADD Research Industries Inc.) in 0.1M PO_4 buffer pH 7.4 for ½-2 hours at room temperature

wash 5 minutes in 0.1M PO_4 buffer pH 7.4 at room temperature

50% ethanol 10-15 minutes

70% ethanol 10-15 minutes

80% ethanol 10-15 minutes

90% ethanol 10-15 minutes

98.5% ethanol two 20 minute changes

Propylene oxide two 15 minute changes

1:1 Propylene oxide: Epon overnight uncovered

Embed in Epon: leave 1-2 hours at room temperature
48 hours in an oven at 60°C

The Epon was prepared as follows: 4 mls of Solution A (80 gm Epon 812 + 91 gm DDSA) + 6 mls Solution B (100 gm Epon 812 - 76 gm NMA) + 0.14 ml DMP-30. All of the embedding chemicals were obtained from LADD Research Industries Inc.

Sections of 500-800 A were made on a Sorval Porter-Blum MT II ultramicrotome using a diamond knife (E.I. Dupont de Nemours & Co. (Inc.)). The sections were picked up on 200 mesh copper grids (Athene type)(LADD Research Industries Inc.) and coated with 0.25% Formvar (LADD Research Industries Inc.). The grids were routinely stained with 2% uranyl acetate (LADD Research Industries Inc.) for 1-2 hours and rinsed in four changes of distilled water. Uranyl acetate was prepared 1 day ahead as a 2% solution in distilled water. The sections were then stained with 0.2% lead citrate (LADD Research Industries Inc.) for 2-5 minutes washed in four rinses of distilled water for a total of 1 minute (the first wash contained 0.01 N NaOH). Lead citrate was prepared by dissolving 0.2 grams of lead citrate in 100 mls of distilled water, 10N NaOH was added drop by drop to the stirring solution until all of the precipitate disappeared.

Fifteen day regenerate irises cultured for 24 hours in medium containing 0.05 mg colchicine/5 mls of medium or 0.02 mg vinblastine/5 ml medium were treated in a manner similar to the controls. Some regenerates which had been

treated with colchicine were implanted in a host eye for three days and were then removed and processed as mentioned previously. Adult newt lenses were also fixed and treated in a similar manner. All electron microscope observations were made on a Philips 200 electron microscope.

E. Fluorescent Antibody Studies

After 15 days *in vivo* culture the corneal incision was reopened with a sharp scalpel and gentle pressure was applied to the anterior part of the eye in order to dislodge the iris implant, which was then carefully removed. The following procedure adapted from Sainte-Marie (1962) was then followed. The tissue was placed in 95% alcohol for 24 hours at 4°C, dehydrated with four 1 hour changes of absolute alcohol; this was followed by three changes 1 hour each of xylene. All of these procedures were carried out at 4°C. The tissues were then allowed to attain room temperature in the final xylene bath. They were passed through four 1 hour baths of filtered paraffin at 56°C and embedded in filtered paraffin. The blocks were stored at 4°C and sectioned at 7 microns on a Spencer 820 microtome. The sections were placed on glass slides previously coated with Mayer's glycerin albumin (BDH). This treatment did not add any non-specific fluorescence to the samples. The slides were stored at 4°C for not more than a week and fluorescent antibody staining was performed according to

Sainte-Marie (1962) as follows:

1. Deparaffination: Slides were deparaffinized in two changes of pre-cooled (to 4°C) xylene, 1 minute each. They were then passed through three 30 second changes of pre-cooled 95% ethanol and through three 1 minute changes of fresh pre-cooled staining buffer (0.118 PO_4 buffer pH 8.4).
2. Fluorescent Antibody Staining: The slides were exposed to absorbed anti-lens specific antibody diluted 1:4 with staining buffer for 30 minutes. They were washed for 10 minutes in staining buffer and then fixed for 20 minutes in pre-cooled 95% ethanol. The slides were washed in three baths of cold staining buffer, 5 minutes each. They were then exposed to fluorescein labelled goat anti-rabbit immunoglobulin (Microbiological Association, Bethesda, Maryland) for 30 minutes. The slides were then washed in three changes of cold staining buffer, 10 minutes each. A drop of glycerol was placed on each slide and a coverslip was gently placed on top. The slides were stored in the dark at 4°C for not more than 2 weeks.

For control purposes, sections of a 15 day regenerate were exposed to serum from a control non-injected rabbit, instead of lens antiserum. Further treatments were as above.

Antibody Preparation

Mature virgin Dutch Belted female rabbits were used. The animals were injected with 2 mls of a homogenate of newt lenses (5 lenses in 1 ml of 0.118 Na l) mixed in 1 ml of Freund's complete adjuvant (Difco Laboratories). Two weeks later the animals were injected with a mixture of 1 ml of antigen (5 lenses per ml) and 1 ml of Freund's incomplete adjuvant. One week later the animals were injected with a mixture of 1 ml antigen (10 lenses per ml) and 1 ml Freund's incomplete adjuvant. Two weeks later the rabbits were bled from the marginal vein of the ear. The blood was left to clot for 1 hour at 37°C. The clot was separated and centrifuged at 4000 g on a Sorval RC 32 centrifuge. The supernatant was removed and centrifuged further at 9,500 g.

Before use the antiserum was absorbed by reacting it with an equal volume of newt tissue powder obtained from lentectomized newts for ½ hour at 4°C. The serum was then tested for reactivity against newt lens extract, by means of immuno-electrophoresis. Glass slides measuring 5 cm. x 12 cm. were thoroughly washed and dried and then coated with 2 mls of .5% Noble agar (Difco). This layer of agar was dried in an oven (60°C) for one hour and the slide was then coated with 10 mls of a 1:1 solution of Noble agar in 2x barbital acetate buffer pH 8.4-86. The 2x barbital acetate buffer was prepared as follows:

10.8 gm sodium barbital

8.6 gm sodium acetate

11.64 ml O.1N HCl

distilled water to 1000 ml.

After the agar had set, a central well 6.5 cm x 1 mm and two circular side wells 1 mm diameter (5 mm from the central well) were cut. The agar was removed from the side wells and 50 µl of a solution of 10 lenses in 0.118 M saline was added to each well. Electrophoresis was then carried out at 4°C for 1½ hours at 5 volts cm on a G.K. Turner electrophoresis cell model 310. After 1½ hours the slides were removed from the electrophoresis chamber, the agar was removed from the central well and the trough was filled with absorbed serum. The slides were carefully placed in a moist chamber at 4°C for 24 hours. After 24 hours any unreacted protein was rinsed from the slides by a further 24 hour wash in 0.1 M PO_4 buffer. The agar was then dried by placing filter paper on its surface for two hours and was then stained for 3 minutes with 0.2% Ponceau S in 3% trichloroacetic acid (TCA). Any unreacted stain was removed by several 1 hour washes in 3% TCA.

Fifteen day regenerate irises that had been incubated for 24 hours in either control medium or medium containing 0.05 mg/plate colchicine and a further fifteen days *in vivo* were used to test for immunofluorescence. Tissue for immunofluorescence was prepared as previously

described. Liver and lens tissues, fixed as described previously, were also used in order to test for the specificity of the serum and the fluorescent staining. Observations of fluorescence were made on a Leitz fluorescence microscope equipped with a high pressure mercury burner. Exciter filters UG1, B612, and U65, were used. The barrier filter was used in position 3 which was the correct setting to observe fluorescein fluorescence. Photographs were made on 35 mm plus-x-pan film using dark-field illumination.

F. Autoradiography

Autoradiography experiments were performed in order to determine whether or not colchicine was inhibiting the incorporation of ^{3}H uridine, ^{3}H thymidine or ^{3}H leucine. (Protein studies also helped to show if crystallin synthesis was being affected.)

In order to determine if nucleotide incorporation was inhibited in my system, 15 day regenerate irises were cultured for 24 hours in control medium or in control medium plus 0.05 mg/plate colchicine. The irises were then implanted into a host newt which was immediately injected with 10 µc of uridine - 5-^{3}H (New England Nuclear) (specific activity 24.9C/millimole) in 50 µl saline or 30 µc of thymidine-methyl-^{3}H (specific activity - 16.9 curies/millimole) (New England Nuclear) in 60 µl saline. The

newts were sacrificed twelve hours later and the eyes were fixed and dehydrated as for light microscopy. The slides were deparaffinized in two 5 minute baths of xylene and then taken through 2 baths of absolute alcohol for 5 minutes each. The slides were then placed in a cold 5% TCA bath for 10 minutes to remove any unbound radioactivity. They were then washed in distilled water, incubated in 10% H_2O_2 (Fisher Scientific) overnight and then washed again in distilled water and air dried. The slides were coated with Kodak NTB-2 emulsion in the dark. They were dried overnight in a neoprene coated test tube rack, placed in a light-tight bakelite slide box and exposed for 3 weeks at room temperature. Slides were developed for 2½ minutes in cold D-11 (Kodak) rinsed once in water and fixed for 5 minutes in Kodak fixer. They were then washed for 20 minutes in running tap water, stained for 2½ minutes in haematoxylin-eosin and mounted in DPX Mountant.

For protein incorporation tests, irises from 10 and 15 day regenerate newts were removed and incubated in either control medium or medium containing .05 mg/5ml of the colchicine. They were implanted into host newts which were sacrificed 0, 5, 10, 15, and 20 days after implantation. Three hours before the animals were sacrificed the newts were injected with 5 µc of D,L,-leucine-4,5-^3H (Sp.ac 35.4 curies/mM) in 50 µl saline (New England Nuclear). The tissues were then processed in the same

manner as the uridine and thymidine incorporation experiments, except that the overnight exposure to 10% H_2O_2 was omitted.

G. Cell Electrophoresis

Changes in cell surface components during lens regeneration have been detected using cell electrophoresis (Zalik and Scott 1972). In an attempt to investigate whether drug treatments had an effect on the cell surface the effect of several enzymes on the electrophoretic mobilities of control and drug treated irises was investigated. Zalik and Scott (1972) found that neuraminidase sensitive groups reappear on the cell surface by 20 days post-lentectomy. Neuraminidase sensitive groups are detected by their method in the normal pigmented iris cell. These groups disappear from the cell periphery as cells dedifferentiate, and reappear again at the onset of redifferentiation which occurs 15-20 days after lentectomy. Twelve irises per experiment were cultured for 24 hours in control media , or in media containing 2.5×10^{-5} M colchicine or 4.4×10^{-8} M vinblastine. They were then cultured for specific time intervals, *in vivo*. The irises were removed from the host newt eyes (for ultraviolet microscopy) and dissociation was performed according to the method developed by Zalik and Scott (1972). The irises were trypsinized with 0.25% filtered purified trypsin

(Fisher Scientific Company) in 0.118 M saline for ½ hour on a shaker waterbath set at 37°C. Cells were recovered by centrifugation at 250 x 9.1 for 5 minutes in an International Clinical Centrifuge. The cells were then washed in 0.118 M saline, and the cell suspension was divided into two tubes. One aliquot of the cell suspension was incubated in an equal volume of saline and the other was incubated at 37°C for 30 minutes in an equal volume of neuraminadase (Behringwerke AG) containing saline (25 units/ml) with 0.005H $CaCl_2$. After incubation, cells were washed in 0.118M NaCl pH 7.2 and used for electrophoretic mobility studies.

The electrophoretic mobility of the cells was measured using a cylindrical cell electrophoresis apparatus (Bangham et al. 1958) equipped with a 1 ml chamber, obtained from Rank Brothers, Bottisham, England. The apparatus consists of an electrophoresis chamber made of a cylindrical glass tube into which platinum electrodes are inserted. The chamber is placed in a thermostated circulating water-bath maintained at 25°C. The electrodes are connected to a power supply and their polarity is reversed between measurements to guard against electrode effects which would result in movement due to drift. A microscope fitted with a water immersion objective lens is immersed in the water-bath and focused on the optical flat surfaces of the glass tube. The cell electrophoresis cell was

calibrated with human erythrocytes, in 0.145 M NaCl pH 7.2. The migration rate of the iris cells was measured by timing their passage over three squares, 15μ each, of a graticule built into the microscope. Therefore each measurement represents the time taken by each cell to travel 45μ. Using this method 3 sets of experiments were performed:

(a) The first set of experiments was done in order to determine when neuraminadase sensitive groups could be detected at the surface of cells from regenerating irises. Irises at 14, 15, 16, 17, 18, and 19 days after lentectomy were used, 12 irises per age. These irises were incubated for 1 day in control medium, and then the effects of neuraminidase treatment on their electrophoretic mobility, (EPM), was determined; (b) It was important to ascertain the necessary *in vivo* incubation time needed for the appearance of detectable neuraminadase sensitive groups at the surfaces of the cells of the irises.For this purpose, 14 day regenerate irises were incubated for 2, 4, 6, and 8 days *in vivo* and then the effect of neuraminadase on the electrophoretic mobility of the cells was determined.

(c) In another series of experiments an attempt was made to investigate whether there was a period of regeneration sensitive to colchicine or vinblastine treatment, which would prevent the reappearance of neuraminadase sensitive groups at the cell surface. Sets of twelve - 8, 10, 13, 14, 15, 16, 18, and 21 day regenerate irises were tested.

The irises were incubated for 1 day in culture medium containing .05 mg/plate colchicine or 0.02 mg/plate vinblastine. They were then incubated for 4 days *in vivo*, cells were obtained, and the effect of neuraminidase on their E.P.M. was studied. The data were analyzed for significance with a paired t-test.

RESULTS

1. Light Microscopy Observations

In order to determine the effect of experimental procedure and drug treatment on the cultured irises a series of criteria were established in order to evaluate the subsequent progress in development of the irises. All histological sections of the cultured regenerates were graded according to the following criteria: (a) ratio of pigmented to depigmented cells in the area making up the regenerate; (b) degree of differentiation of primary lens fibers. Primary fibers were divided into three subclasses: I. beginnings of cell elongation with nuclear and cytoplasmic basophilia still apparent, II. increased cell elongation and beginnings of cytoplasmic acidophilia, III. intense cytoplasmic acidophilia,increased lens fiber elongation and onset of nuclear degeneration: these fibers will eventually be located in the central core of the mature lens. During depigmentation and early stages of lens fiber formation the cytoplasm of the cells becomes basophilia due to increased production of ribosomes; as lens proteins are synthesized in the cytoplasm the basophilia gradually decreases and is replaced by acidophilia,(c) presence of secondary lens fibers. Secondary lens fiber cells appear between the depigmented cuboidal cells of the

regenerate and the elongating primary lens fiber cells as the primary fiber cells begin to form the central core of the lens. According to these observations an estimate of the stage of regeneration was made. The staging system proposed by Yamada was used in these studies. From the relationship between the average time after lentectomy and the incidence of certain regeneration stages at that time during normal regeneration, it was possible to estimate the apparent age in time of the regenerate according to the degree of regeneration it had attained, i.e. a Stage V regenerate would occur between 12-15 days after lentectomy, or an average of 13.5 days. In order to determine if any retardation occurred due to experimental conditions or drug treatment, the apparent age (in days post-lentectomy) of each sectioned regenerate was estimated. This was compared to the actual age of the regenerate and was used as an index of the extent of retardation.

A. Retardation Due to Experimental Conditions - Intrinsic Retardation

The methods chosen for treatment of the iris with certain drugs were used because one can place the regenerate in a medium with a known drug concentration, and because secondary drug effects on the experimental animals which may in turn influence the system under investigation, are minimized. They also enable the investigator to maintain *in vivo* conditions for most of the duration of the

experiment. However, they do have a drawback, the iris is subject to certain damage during microsurgery; therefore it is important to establish the extent of retardation that is introduced into the system by mechanical manipulation and control culture conditions. Table 1 is a summary of data collected from various age irises that were surgically removed, cultured for 24 hours *in vitro* under control culture conditions and subsequently implanted into host eyes for 15 days. Table II summarizes the calculations required to obtain the intrinsic retardation experienced by the different age irises. Figure 3 plots the data obtained from Table 2. As can be seen in Figure 3, there is an intrinsic retardation introduced by experimental manipulation which increases linearly as the postlentectomy age of the treated irises increases .

B. Effects of Colchicine on Lens Regeneration

1. Dose Response

This series of experiments was performed on 15 day regenerates in order to find a dose of colchicine which would inhibit elongation without being lethal to the cells. Irises at 15 days postlentectomy were used because at this age cells start to elongate and differentiate into primary lens fibers. The results obtained from these experiments are presented in Table III. From these data it can be seen that all of the doses of colchicine that were tested

Table I

Data Describing a Series of Control Irises

Irises were cultured for 24 hours in L-15 medium (50% V/V) and implanted into the eye of a host lentectomized newt for 15 days.

x - Age refers to the number of days postlentectomy.

xx - Usually more than 50% of the treated irises were unsatisfactory for study due to improper implantation in the eye, infection, loss during sectioning or mechanical damage to the regenerate. This column represents the number of irises tested, the number that were used to make observations can be seen in the other columns.

xxx - Ratio of depigmentation is calculated for the area making up the regenerate.

+ - 0-25% depigmented cells/pigmented cells

++ - 25-50% depigmented cells/pigmented cells

+++ - 50-70% depigmented cells/pigmented cells

++++ - 75-100% depigmented cells/pigmented cells

xxxx - Primary lens fiber cells were divided into three subclasses:

I beginnings of cell elongation with nuclear and cytoplasmic basophilia still apparent.

II increased cell elongation and beginnings of cytoplasmic acidophilia

III increased lens fiber elongation, onset of nuclear degeneration, usually these fibers will be present in the central core of the mature lens.

Table I

Data Describing a Series of Control Irises

Age[x]	No. Tested[xx]	Ratio of Depigmentation[xxx]	Primary Lens Fiber Cells I	II	III	Secondary Lens Fiber Cells	Stage
0	10	++++	x	x	x	-	VI
		"	x			-	IV
		"	x	x		-	VI
		"	x			-	V
2	10	++++	x			-	IV
		"	x	x		-	VI
		"	x	x		-	VI
		"	x			-	IV
5	10	++++	x			-	V
		"	x	x		-	VI
		"	x	x		-	VI
		"	x	x		-	VI
6	10	++++	x	x		-	VI
		"	x	x		-	VIII
		"	x	x		-	VII
		"	x	x		-	VII
		"	x	x		-	VI
7	10	++++	x	x		-	VIII
		"	x	x		-	VII
		"	x	x		-	VIII
		"	x	x		-	VIII
		"	x	x		-	VIII

Age[x]	No. Tested	Ratio of Depigmentation[xxx]	Primary Lens Fiber Cells I	II	III	Secondary Lens Fiber Cells	Stage
10	20	++++	x	x	x		VIII
		"	x	x			VII
		"	x	x			VIII
		"	x	x			VIII
		"	x	x	x		VIII
		"	x	x	x		VIII
		"	x	x	x	x	IX
		"	x	x			VIII
15	20	+++	x	x			VII
		"	x	x	x		VIII
		"	x	x	x		VIII
		"	x	x	x	x	IX
		"	x	x	x	x	IX
		"	x	x	x	x	IX
		"	x	x	x	x	IX
		"	x	x	x	x	X
20	10	++++	x	x	x	x	X
		"	x	x	x	x	X
		"	x	x	x	x	X

TABLE II

Summary of Calculations for Intrinsic Retardation of Control Irises

x - actual age was calculated by adding the number of days postlentectomy plus 1 day for *in vitro* culture plus 15 days *in vivo* culture.

xx - The apparent age was estimated by matching the observed stages with stages that occur during normal regeneration. The normal regeneration stages have a sequence of appearance at specified time intervals. These times were used as the apparent age for the stages reached under experimental conditions.

xxx - The average apparent age in this column is not necessarily the numerical average of the range of apparent ages. It is calculated for the group of irises tested, i.e. for 0 days postlentectomy there were 2 stages VI's, 1 stage V and 1 stage IV irises, therefore instead of the average apparent age being 13 it was 14 because the 2 stage VI's weighed the average.

xxxx - Intrinsic Retardation was obtained by subtracting the apparent age from the actual age.

Table II

Summary of Calculations for Intrinsic Retardation of Control Irises

Days Postlentectomy	Actual Age	Stages	Apparent Age/ Average Apparent Age	Intrinsic Retardation
0	16	IV-VI	10-16/14	2
2	18	IV-VI	10-16/14	4
5	21	V-VI	12-16/15	6
6	22	VI-VIII	15-18/16	6
7	23	VII-VIII	15-19/17	6
10	26	VII-IX	16-20/18	8
15	31	VIII-IX	18-25/20	11
20	36	X	18-25/22	14

Figure 3

Graph of the Intrinsic Retardation Experienced by Various Age Regenerates

The data and calculations from Tables I and III were used to construct this graph. There appears to be a linear relationship between intrinsic retardation and the age, postlentectomy of the iris that is studied. The more advanced the age of the regenerate postlentectomy, the greater the intrinsic retardation.

Each point on the graph represents an average figure calculated from seven or more irises .

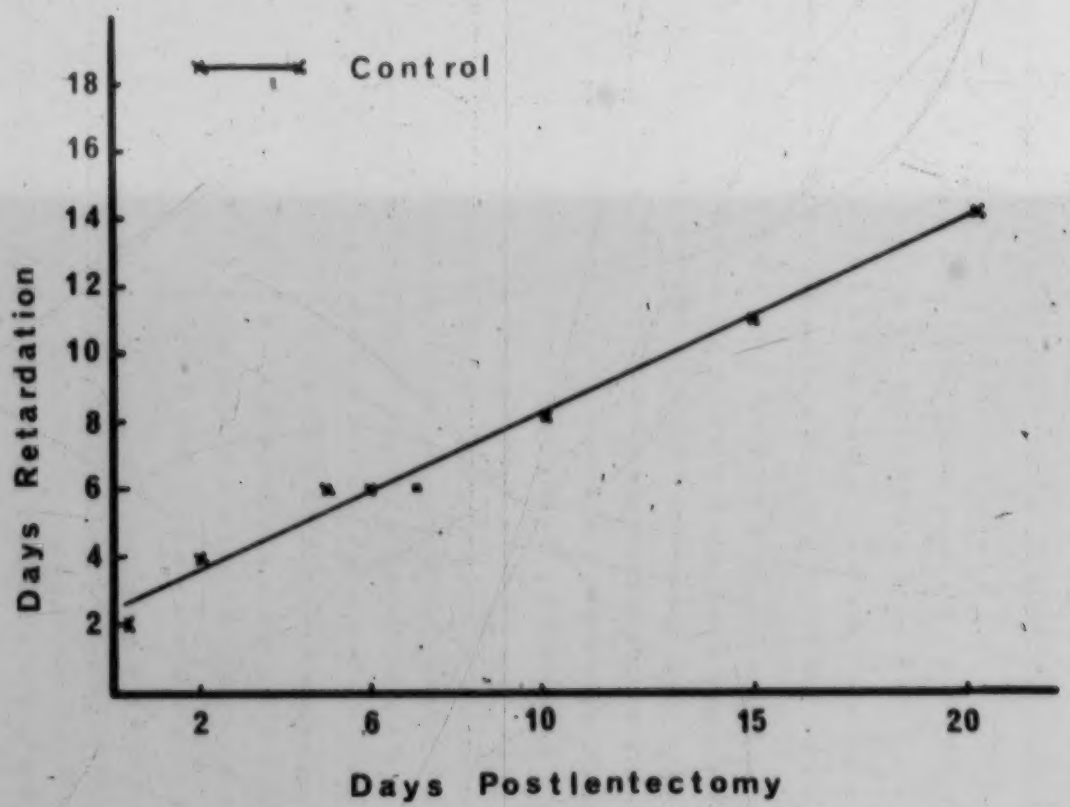

Control
18
16
14
12
10
8
6
4
2
Days Retardation
2
6
10
15
20
Days Postlentectomy

Table III

Summary of Data for Dose Response to Colchicine

Treatment	Dose	No. Observed/ No. Attempted	Stage	Apparent Age/ Average Apparent Age	Actual Age	Retardation
Control	-	4/5	VIII-IX	18-25/20	31	11
Colchicine	2.5×10^{-6} M	3/5	VI-VII	12-18/16	31	15
Colchicine	2.5×10^{-5} M	3/5	V	12-15/13	31	18
Colchicine	2.5×10^{-4} M	3/5	V	12-15/13	31	18
Colchicine	1.25×10^{-3} M	2/5	III	8-11/10	31	21

For this experiment 15 day regenerate irises were treated for 24 hours and then incubated *in vivo* for 15 days therefore the actual age was 31 days.

retarded regeneration. Colchicine at 2.5×10^{-6} M retarded regeneration but it allowed cellular elongation and lens fiber formation to occur as evidenced by the fact that stages VI-VII, characterized by the presence of elongated primary lens fibers, were attained. However, elongation was inhibited at colchicine concentrations of 2.5×10^{-5} M to 1.25×10^{-3} M. At these concentrations the regenerate only developed up to stage V. This stage is characterized by an internal and external layer of depigmented cells that exhibit no elongation. None of the doses seemed to be lethal to the cells of the regenerate since on histological examination all of the cells appeared healthy. There was no morphological evidence of cytoplasmic or nuclear damage. Moreover if regenerates were cultured for longer time intervals *in vivo* they recovered and were able to reach later stages of regeneration (see section 2). For subsequent work a dose of 2.5×10^{-5} M was selected since this was the lowest dose which would effectively inhibit cell elongation.

On histological examination the control regenerates appeared as follows: in many there was a lens epithelium of cuboidal cells around an organized lentoid. Elongating primary lens fiber cells were present and in some cases secondary fiber cells also occurred. Colchicine treated 15 day regenerates cultured for 15 days *in vivo* often appeared as an unorganized mass of depigmented cells showing no signs of elongation. In a few regenerates

some organization into internal and external layers was evident but no signs of fiber elongation were present. See Figure 4 for an illustration of control and colchicine treated irises.

2. Most Effective *in vivo* Incubation Time

These experiments were done in an attempt to find an incubation time which would most show the effects of colchicine. Results of these experiments are summarized in Table IV and Figure 5. Ten irises per experiment were tested, and about 5 out of the 10 irises could be used for the observations. From these results it can be observed that 15 days of *in vivo* incubation was the time interval at which colchicine treatment produced the greatest extent of retardation. Incubation periods of 5 and 10 days already showed inhibition of cell elongation; however differences between controls and experimentals were not as pronounced. This suggests that it may take drug treated and control irises several days to recover from the operational techniques before experimental differences become evident. As illustrated in Figure 6 incubation times longer than fifteen days for control irises result in an increase in size of the regenerate, and increase in the number of primary lens fibers. There is also an increase in the number of secondary fibers and the size of the central core - similar to what occurs *in*

Figure 4

Micrographs of 15 day Regenerate Irises Treated with Colchicine or Control Medium for 1 Day *in vitro* and then Incubated 15 Days *in vivo*

A - A Stage X control iris. Primary (P) and secondary (S) fibers are present . x 125

B - A Stage X control iris. Primary (P) and secondary (S) fibers are present . x 125

C - A Stage VI colchicine treated iris. The beginnings of elongation are present in a few cells. (The elongating cells are indicated by an arrow.) x 125

D - A Stage V colchicine treated iris. This regenerate is a mass of depigmented cells. The depigmented cells are indicated by arrows. x 125

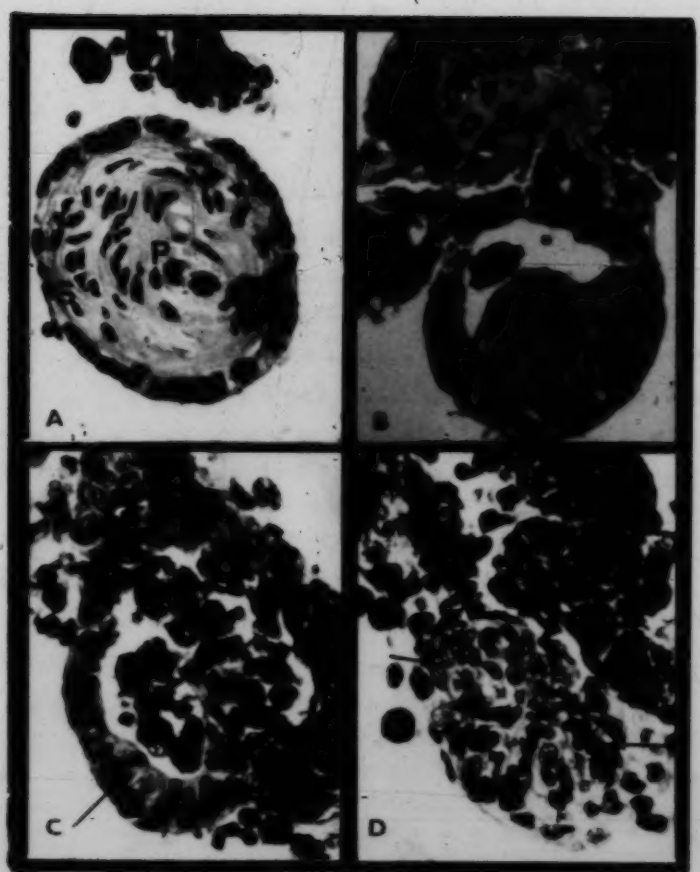

P
A
B
C
D

Table IV

Retardation Rate of 15 Day Regenerates at Different Time Intervals after Culture in Host Lentectomized Eyes

Incubation Time (Days)	Control Stages	Control Age/Average	Actual Age	R1	Stages	Age/Average	R2	R2-R1
5	V-VII	12-18/15	21	6	V	12-15/12	9	3
10	VII-IX	16-20/18	26	10	V	12-15/12	13	5
15	VII-X	15-25/20	31	11	V-VI	12-16/14	17	6
20	X	18-25/22	36	14	VI-VII	12-18/17	19	5
25	X	21-25/24	41	17	VIII-IX	15-20/19	22	5

*R1 - Retardation in days experienced by the control due to experimental procedures - intrinsic retardation

**R2 - Retardation in days experienced by the colchicine treated irises due to experimental conditions and colchicine treatment

***R2-R1 - Retardation due to the effect of colchicine alone

Figure 5

Graph of Colchicine Retardation Versus
in vivo Incubation Time

This graph shows that an incubation time of 15 days *in vivo* produced the greatest retardation of colchicine treated 15 day regenerates. After 15 days incubation, irises showed some elongation and appeared to be developing more rapidly.

Each point on the graph represents an average retardation time for 5 irises.

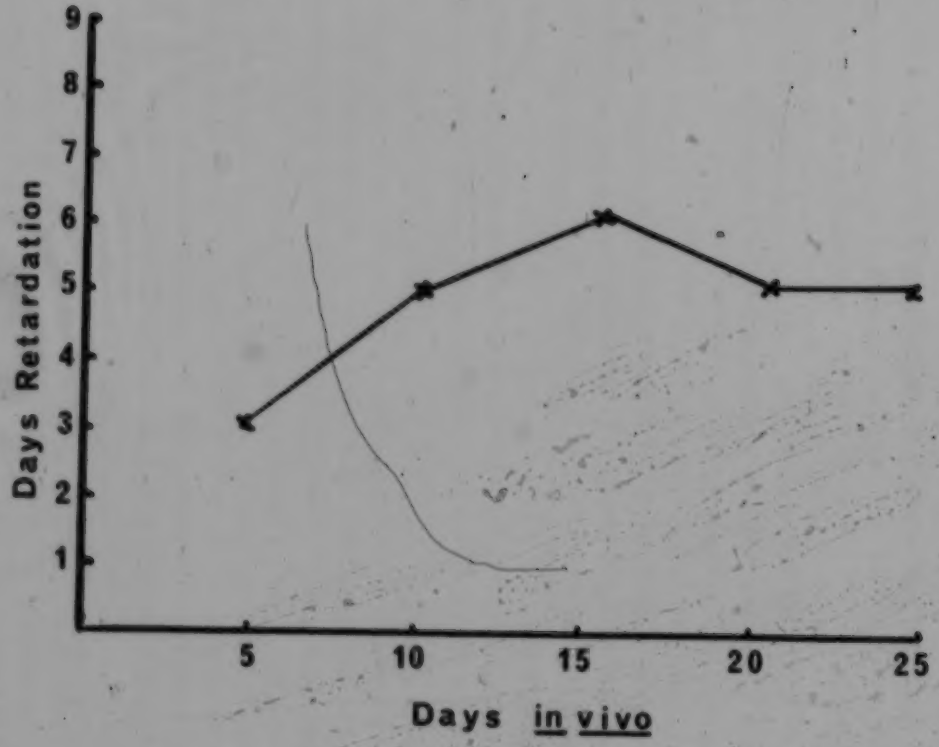

Days Retardation
9
8
7
6
5
4
3
2
1
5
10
15
20
25
Days in vivo

Figure 6

Micrographs of 15 Day Postlentectomy Regenerates Incubated for 1 Day *in vitro* and Varying Times *in vivo*

A - A Stage VIII control iris incubated for 10 days

x 125

B - Stage X regenerate which was incubated for 15 days

x 125

C - Stage X control iris incubated for 20 days

x 125

The position of the cornea of the host eye in which the regenerates were incubated in is indicated by an arrow.

A
B
C

situ. Figure 7 illustrates that colchicine treated regenerates are able to overcome the inhibition of elongation. Up to 15 days *in vivo* incubation most of the regenerates were halted at Stage V, whereas after more than 15 days in culture they are able to attain later stages of regeneration.

3. Most Sensitive Stage

Preliminary experiments on dose response and *in vivo* incubation time used 15 days regenerate irises since this was the most probable time at which the onset of elongation was occurring. This set of experiments attempted to find out whether colchicine affected other stages of regeneration. Results from these experiments are summarized in Table V and are shown in the form of graphs in Figures 8 and 9. It can be seen from Figure 9 which plots the actual retardation of the implant due to colchicine treatment, that a 15 day regenerate is most sensitive to colchicine treatment, or at least it demonstrates the most retardation relative to the controls.

As seen in Table V irises treated at 0 and 2 days postlentectomy show no observable inhibition due to colchicine treatment. As later stage regenerates (5-15 days postlentectomy) are treated there appears to be a progressive increase in inhibition. Careful observation of the data shows that all of the regenerates treated with colchicine from 5-15 days (prior to the onset of

Figure 7

Micrographs of 15 Day Regenerate Irises Incubated for 1 Day *in vitro* and Varying Times *in vivo*

A - Control--a control iris, incubated for 15 days *in vivo*. Notice the primary and secondary lens fibers indicated by p and s respectively. x 200

B - A Stage V colchicine treated iris incubated for 15 days *in vivo*. No primary lens fibers are observable. x 125

C - A Stage VI colchicine treated iris incubated for 20 days *in vivo*. Primary lens fibers have started to form. They are indicated by P. x 125

D - A Stage IX colchicine treated iris incubated for 25 days *in vivo*. This was the most advanced colchicine treated iris observed - primary and secondary fibers are present. They are indicated by P and S respectively. x 125

An arrow indicates the position of the cornea of the host eye in which the regenerates were cultured.

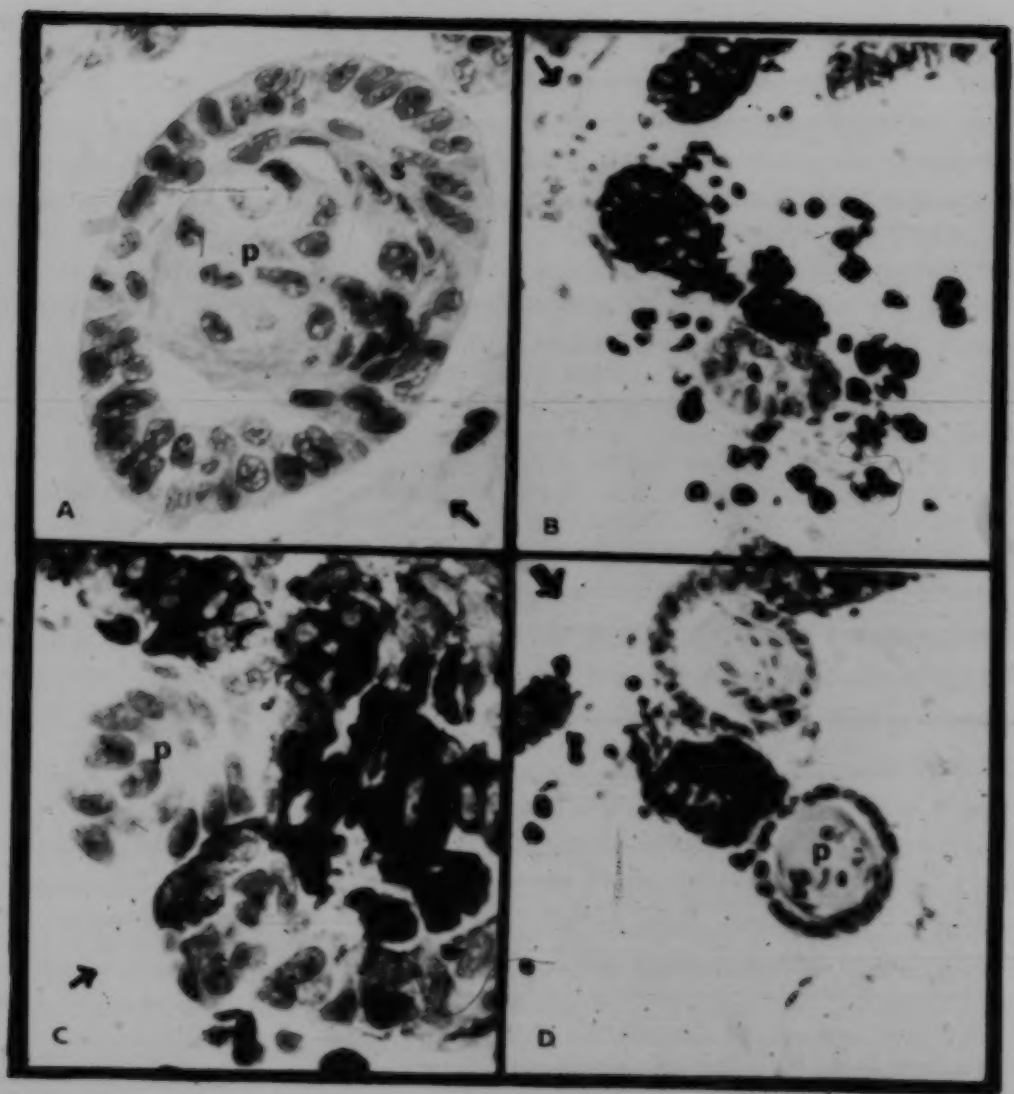

p
A
B
p
C
p
D

Table V

Operated Controls Compared to Operated Colchicine Treated Experimentals

Age of Iris After Lentectomy	Total Age	Stage of Operated Control/ Av. Apparent Age	Stage of Colchicine Treated/ Av. Apparent Age	R1*	R2**	R2-R1***
0	16	IV-VI/14	IV-VI/14	2	2	0
2	18	IV-VI/14	IV-VI/14	4	4	0
5	21	VI/15	VI/14	6	7	1
6	22	VI-VIII/17	V-VI/14	5	8	3
7	23	VII-VIII/17	V/13	6	10	4
10	26	VII-X/18	V-VI/14	8	12	4
15	31	VII-X/19	V-VI/14	12	17	5
20	36	X/22	VII-VIII/18	14	18	4

*R1 - operational retardation - (Total age - av. apparent age of control)

**R2 - operational plus colchicine retardation (Total age - av. apparent age of colchicine)

***R2-R1 - Colchicine retardation

Figure 8

Graph of the Retardation Experienced by Different Aged Regenerates Due to Colchicine Treatment and/or Experimental Conditions

Each point on the graph represents an average retardation time for at least 5 irises . These points were plotted from the data presented in columns R1 and R2 in Table V .

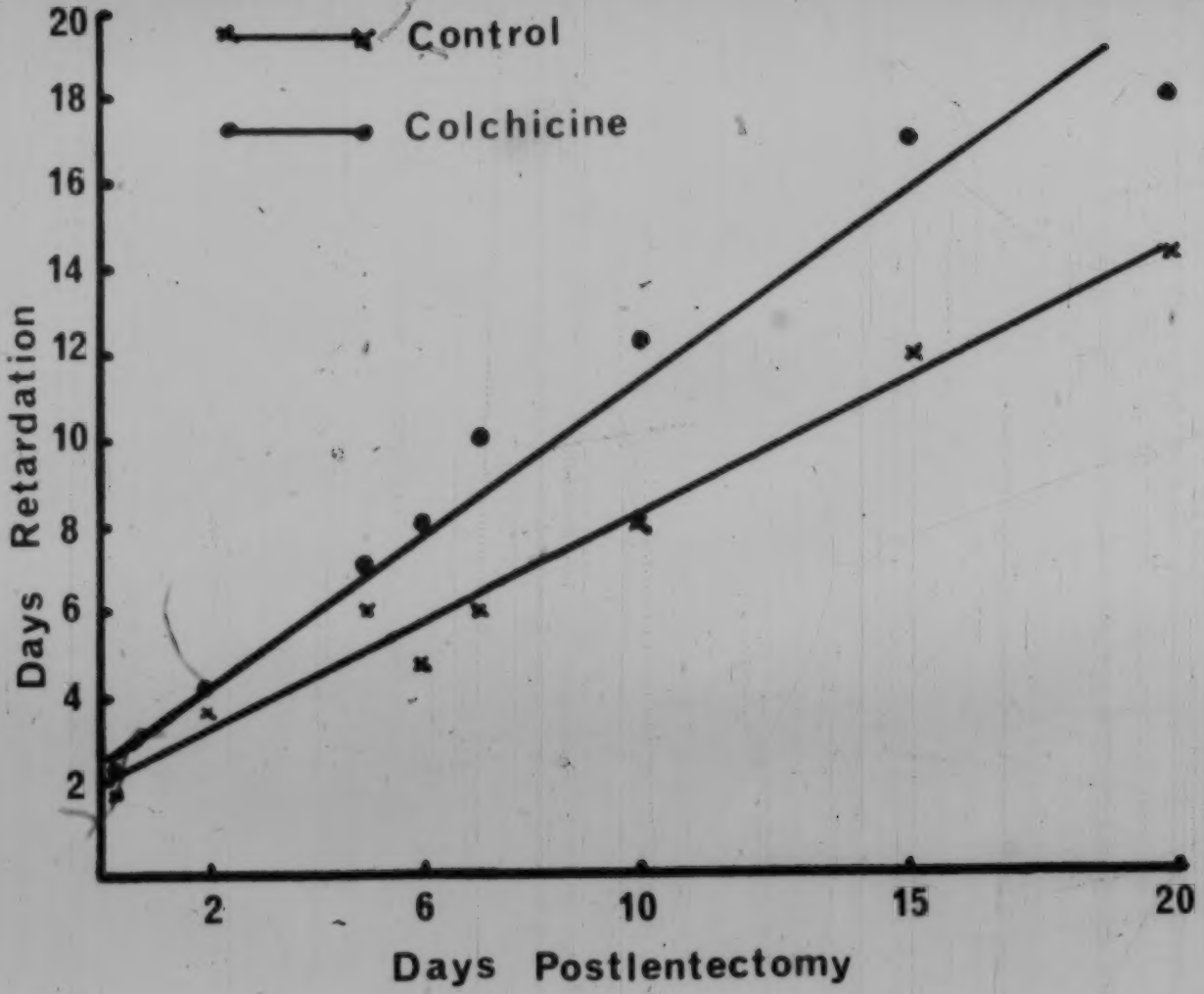
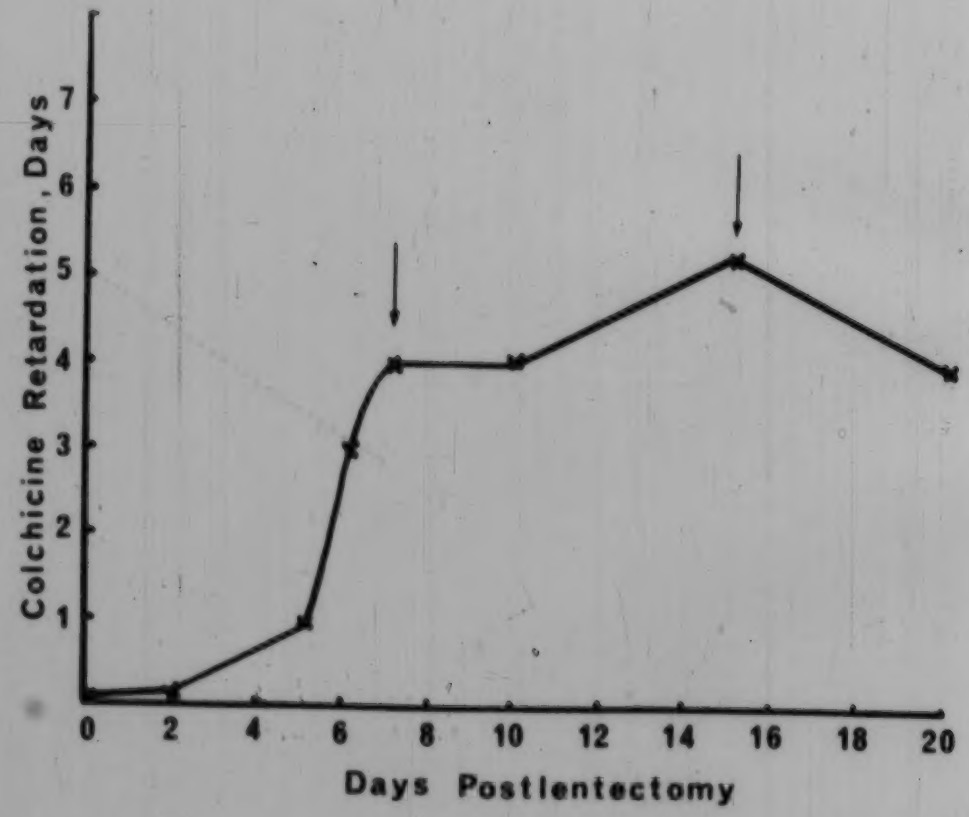

Control
Colchicine
Days Retardation
20
18
16
14
12
10
8
6
4
2
2
6
10
15
20
Days Postlentectomy

Figure 9

Graph of the Retardation of the Iris Due Solely to Colchicine Treatment versus the Age of the Treated Regenerate in Days

The arrows indicate the times at which peaks of mitosis appear (according to Yamada and Roesel 1971). It can be seen that there is more relative retardation at the times when the mitotic peaks occur ; however, there is more retardation in a 15 day regenerate than in a 7 day regenerate . This suggests that some process other than mitosis is being affected .


Colchicine Retardation, Days
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18
20
Days Postlentectomy

elongation) are halted at Stage V or VI - stages at the onset of cellular elongation. Irises treated at 20 days postlentectomy, an age at which cellular elongation has already occurred, show a delay in development; however, they were able to attain Stages VII-VIII which are characterized by the presence of elongated fiber cells. It therefore appears that once cellular elongation has occurred it is not reversed by colchicine treatment. In Figure 4 it can be seen that while extensive cellular elongation and primary lens fiber formation has occurred in the control cultured 15 day regenerates, colchicine treated regenerates at this stage show little, if any, cell elongation. Five day regenerates are shown in Figure 10. The onset of cellular elongation is evident in the control, whereas none is apparent in the colchicine treated regenerate. Figures 11 and 12 illustrate the greater differences apparent between colchicine and control treated regenerates in 7 day regenerates. The controls show well-organized lentoids - some even show evidence of secondary fiber formation. The colchicine treated regenerates are for the most part masses of depigmented cells although Figure 12c, shows that some cellular elongation has taken place. Figure 13 illustrates the effects of colchicine and control treatment on 10 day regenerates. The control regenerate shows elongating primary fiber cells whereas the colchicine

Figure 10

Micrographs of 5 Day Regenerates Incubated for Fifteen Days *in vivo* after 1 Day Incubation *in vitro*

A - A Stage VI control treated iris. The arrow indicates a region beginning to show elongation. x 125

B - A Stage V colchicine treated iris. There does not appear to be any elongation anywhere in this lentoid. x 125

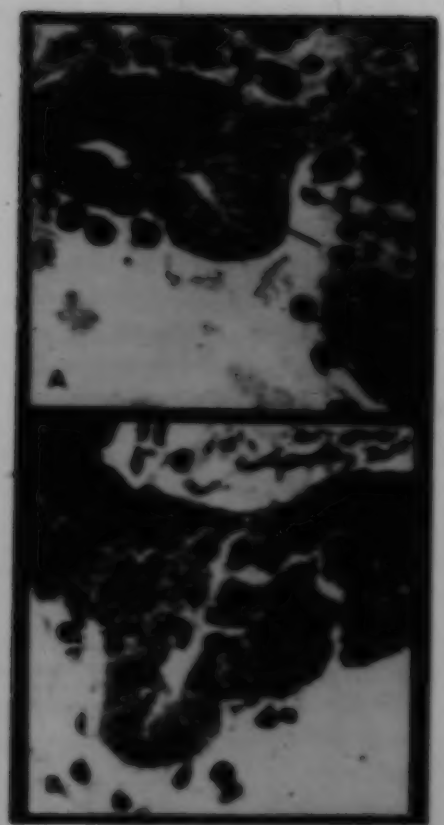


Figure 11

Micrographs of 7 Day Regenerate Irises Incubated for 1 Day *in vitro* and 15 Days *in vivo*

A - A Stage VII control iris which shows primary lens fibers. x 125

B - A Stage VII control iris. x 125

C&D - Stage V colchicine treated regenerates. Both of these regenerates appear as organized masses of depigmented cells, however neither show any elongation. x 125

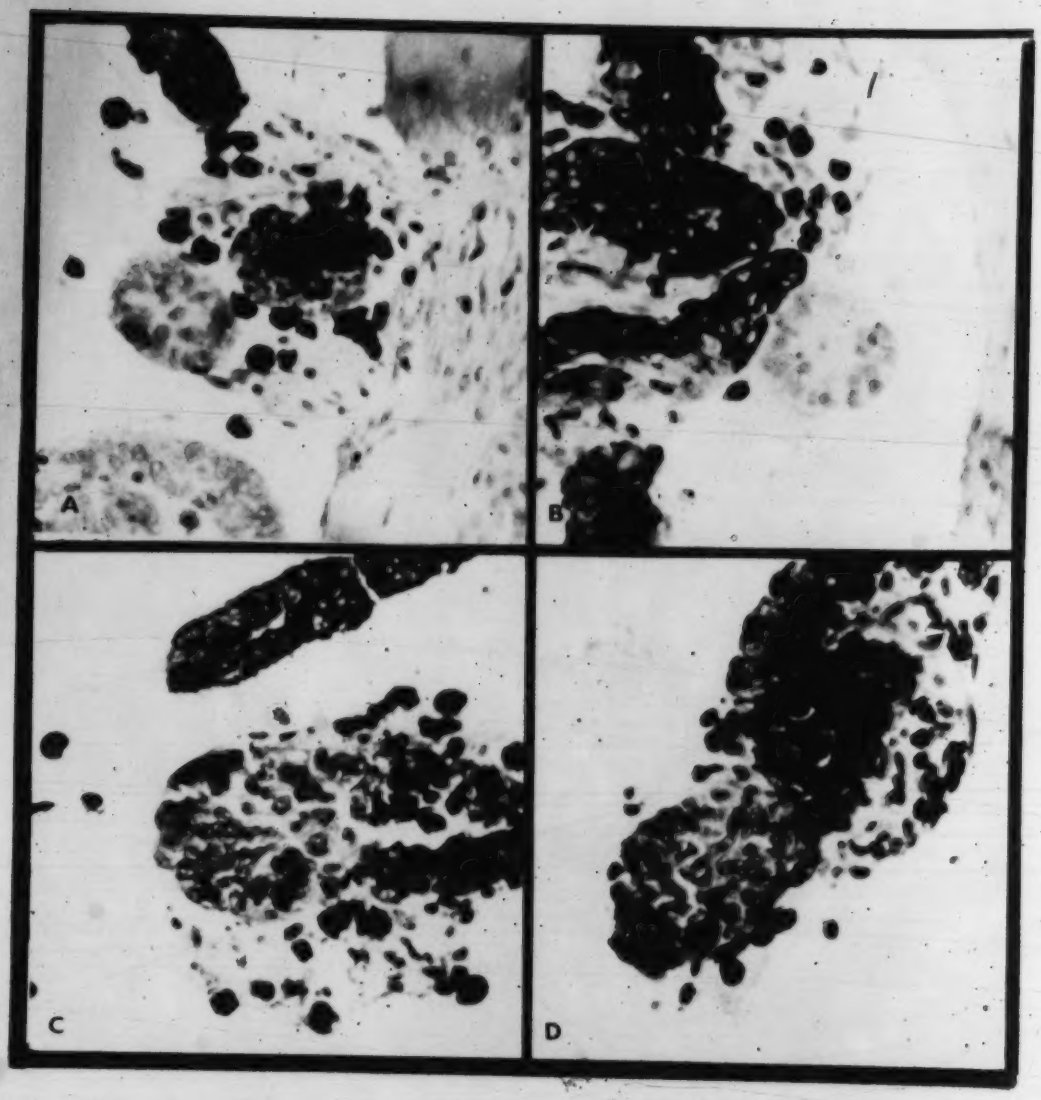

A
B
C
D

Figure 12

Micrographs of 7 Day Regenerate Irises Incubated for 1 Day *in vitro* and 15 Days *in vivo*

A & B - Control irises which have attained Stage VII. Notice onset of elongation in the inner wall of the regeneration. x 125

C - A Stage VI colchicine treated iris. A few of the cells show the beginnings of elongation (arrow). x 125

A
B
C

Figure 13

Micrographs of 10 Day Regenerate Irises Incubated for 1 Day *in vitro* and 15 Days *in vivo*

A - A Stage VII control iris showing primary lens fibers (arrow) x 125

B - A Stage V colchicine treated regenerate . There appear to be two patches of depigmented cells in this regenerate - indicated by arrows. x125

C - A Stage V colchicine treated regenerate. This regenerate has assumed an abnormal shape. It is composed of a mass of depigmented cells. x 125

A
B
C

treated regenerates appear as masses of depigmented cells without any apparent elongation.

4. Effects of Colchicine on the Incorporation of ^{3}H-Uridine, ^{3}H-Thymidine and ^{3}H-Leucine

It has been reported (Hell and Cox 1963, and Creasey and Markiw 1965) that colchicine can inhibit nucleotide incorporation; therefore, a series of experiments were done in order to determine if colchicine was causing an inhibition of ^{3}H-uridine and ^{3}H-thymidine incorporation in the drug treated regenerates under my experimental conditions.

(a) Incorporation of ^{3}H-Uridine

On histological examination of sections of control and colchicine treated regenerates there did not appear to be any difference in ^{3}H-uridine incorporation. Grain counts made over areas of similar size are presented in Table VI. There does not appear to be any difference in the number of counts per unit area when colchicine and control regenerates are compared. This indicates that there is no appreciable inhibition of ^{3}H-uridine incorporation by colchicine.

Table VI

Incorporation of ^{3}H-Uridine into a 15 Day Regenerate

^{3}H-Uridine Incorporation
Counts per Implant

Control	Colchicine
40	50
45	35

b) Incorporation of ^{3}H-Thymidine

Sections of similar area were also used for counting in the experiment. Since under the conditions employed in these experiments there was a heavy incorporation of ^{3}H-thymidine into the regenerate nuclei, resulting in too many grains to be accurately counted, the total number of nuclei in representative sections was counted and then the number of labelled nuclei was counted. The results are summarized in Table VII.

Table VII

Incorporation of ^{3}H-Thymidine into a 15 Day Regenerate

^{3}H-Thymidine Incorporation

Number of labelled nuclei/Total number of nuclei

Control	Colchicine
8/30	12/40
4/20	

The data indicate that there is a similar ratio of cells in DNA synthesis in control and colchicine treated regenerates.

c) Incorporation of ^{3}H-Leucine

Yamada and Takata (1963) have shown that during the course of lens regeneration there is a sharp increase in protein synthesis occurring at about 15 days postlentectomy. This has been assumed to correspond to the onset of lens crystallin synthesis in elongating fiber cells. In order to investigate whether colchicine inhibits the onset of this increase in protein synthesis, studies of ^{3}H-leucine incorporation were done. Since increased protein synthesis occurs first in a localized number of cells within the regenerate, it would be difficult to get an accurate estimate of protein synthesis by randomly selecting an area and making grain counts. Therefore grain counts were made by selecting three cells that seemed to have a relatively large number of grains; these were counted and called X. Similarly three cells with a small number of grains were counted and called Y. The sum of X + Y was compared for the various tests on control and colchicine treated irises. These results are summarized in Table VIII and Figure 14. This method of counting was used so that the localization of high counts in a few cells of the regenerate would be immediately apparent by comparing X + Y for each experiment. A comparison of the

Table VIII

Incorporation of ^{3}H-Leucine into 10 and 15 Day Regenerates Incubated for Varying Periods of Time *in vivo*

*X - the sum of counts for three cells which showed heavy labelling.

*Y - the sum of counts for three cells which showed light labelling.

The arrows indicate where there were instances of heavy labelling in a few cells of the regenerate and very little labelling in the rest of the regenerate .

Table VIII

Incorporation of ^{3}H-Leucine into 10 and 15 Day Regenerate Irises Incubated for Varying Periods of Time *in vivo*

10 day regenerate

Days Incubated *in vivo*	COUNTS Control X* + Y**	Total	Colchicine X + Y	Total
5		5		5
10	47 + 38	85	12 + 11	23
	50 + 40	90		
	42 + 32	74		
15	37 + 31	68	31 + 28	59
	42 + 32	74	40 + 33	73
	48 + 40	88	63 + 38	101
	44 + 36	80		
	54 + 50	104		

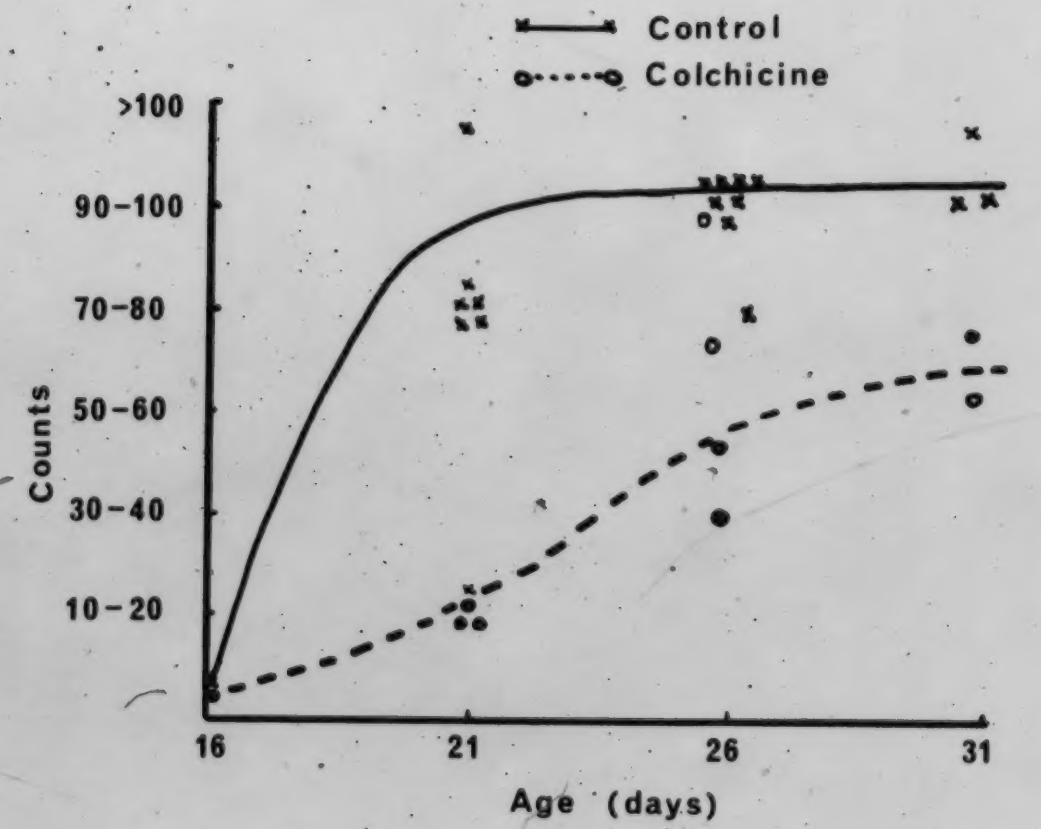
15 day regenerate

0		4		4
5	44 + 33	77	10 + 9	19
	→ 76 + 16	92	11 + 7	18
	→ 150 + 22	177		
10	49 + 41	90	29 + 21	47
	42 + 38	80	29 + 28	57
	51 + 45	96		
15	56 + 29	85	38 + 31	69
	72 + 46	118	43 + 38	81
	49 + 39	88		

Figure 14

A Graph of Total Counts of ^{3}H-Leucine Incorporation versus total Age of the Regenerate

*Counts refer to total of X + Y as referred to in Table VIII


Control
Colchicine
>100
90-100
70-80
50-60
30-40
10-20
Counts
16
21
26
31
Age (days)

totals shows that increases in protein synthesis occurred at about 21 days post-lentectomy. By 25 days, all the elongating cells of the lentoid show a sharp increase in protein synthesis. A similar pattern was not observed in the cells of colchicine treated regenerates. Although the latter showed an increase in protein synthesis, the increase was not as great as that observed in the controls.

Takata *et al* (1964) have shown with fluorescent antibodies that crystallin synthesis appears first in a few cells of Stage IV (9-15 days) regenerates and then spreads out and occurs in all of the elongating cells of the regenerate. Since my experimental system has an intrinsic retardation of 8 days (for a 10 day control regenerate iris), and 11 days (for a 15 day control regenerate iris), the results on the control support the findings of Takata *et al* (1964) for normal regeneration. In the studies of these investigators the onset of crystallin synthesis occurs at 9-15 days postlentectomy. In the *in vivo* culture system studied here this onset occurs at 21 days, - a retardation of 6-12 days which agrees with my calculated intrinsic retardation for this system. Colchicine does not seem to completely inhibit protein synthesis at any time.

5. Effects of Colchicine on the Synthesis of Lens-Specific Protein

(a) Specificity of the Antiserum

Reaction of the antiserum with an extract of newt lens after immunoelectrophoresis revealed three bands (see Figure 15). These three bands correspond to bands for α, β and γ crystallins. The most anodic band represents gamma crystallins and the most cathodic represents alpha crystallins. Beta crystallins correspond to the intermediate band (Clayton 1970). Therefore this serum contains antibodies against all of the main crystallins. Newt liver tissue, reacted with antiserum and stained subsequently with fluorescein conjugated anti-rabbit immunoglobulin showed no detectable fluorescence.

(b) Reaction of the Antiserum with Control and Colchicine Treated Irises

In order to determine whether inhibition of elongation in the cells of the regenerate was accompanied by inhibition of lens-specific proteins, fluorescent antibody studies were performed on 15 day regenerates cultured 1 day *in vitro* in the presence or absence of colchicine and 15 days *in vivo*. Figure 16 shows the results obtained when regenerates treated with absorbed anti-lens serum were stained with fluorescein conjugated anti-rabbit inmunoglobulin. Control irises fluoresced very brightly. Fluorescence was present in all of the lens fiber cells, some fluorescence was present in the lens epithelum

Figure 15: Immunoelectrophoresis of lens versus lens specific antiserum

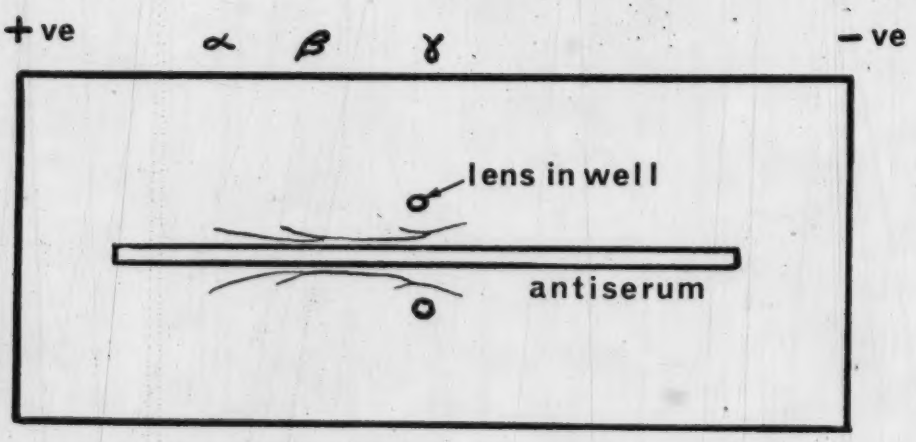


Figure 16

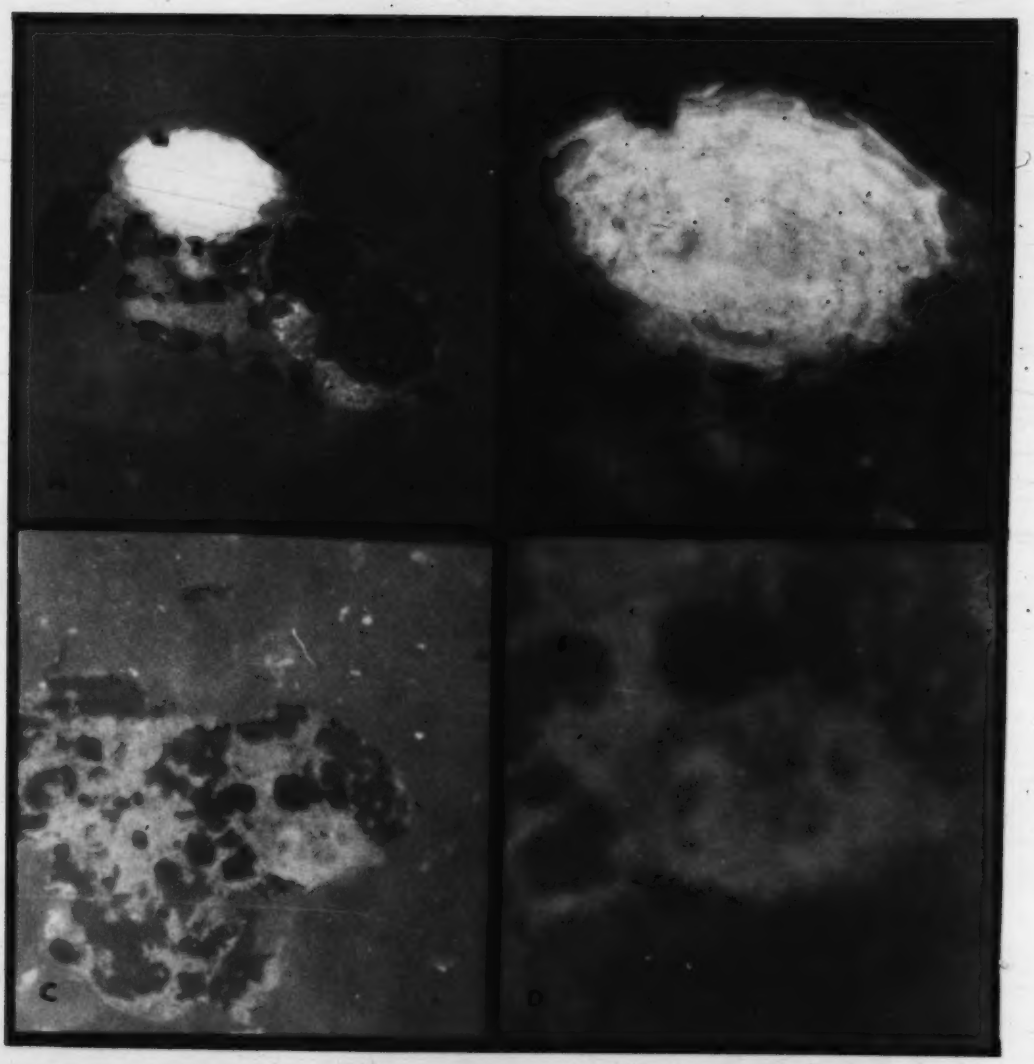
Micrographs of Fluorescein Treated 15 Day Regenerates Incubated for 1 Day *in vitro* and 15 days *in vivo* and Observed under Ultra-violet Light

a - A control treated regenerate treated with fluorescein isothiocyanate. Note the fluorescence present in all areas of the lentoid. Fluorescence is less in the lens epithelium (arrow). x 125

b - An enlargement of (a). Note the reduced fluorescence in the lens epithelium. x 400

c - A colchicine treated regenerate. Note the absence of any fluorescence in the lentoid. x 125

d - An enlargement of (c). x 400


C
D

although it was not as bright as that in the fiber cells. No fluorescence was present in other tissues of the eye. Colchicine treated irises showed almost no fluorescence. One or two tiny spots of fluorescence were present in a few of the internal cells of one of the lentoids. Five irises were examined for both control and colchicine treated regenerates - a lentoid was present in 4 of the 5 controls and 3 of the 5 colchicine treated regenerates.

Tests with serum from an uninjected rabbit showed no fluorescence in control lens regenerates.

c) Effects Due to Lumicolchicine

Linskens and Wulf (1953) and Wilson and Friedkin (1967) have shown that lumicolchicine, a derivative of colchicine, has no mitotic activity nor does it bind to extracts of the mitotic spindle as does colchicine. Others (Berlin 1973) have shown that while lumicolchicine may not have antimitotic properties it does exhibit other properties of colchicine not presumably associated with microtubular function, i.e. inhibition of nucleotide uptake. Therefore experiments were performed with lumicolchicine to determine if this compound had any effects on cell elongation under my experimental conditions.

It was found that lumicolchicine treated regenerates appeared similar to the controls - there was no inhibition of elongation .

D. Effects Due to Vinblastine

(a) Dose Response

First series of experiments were run with 15 day regenerates in order to determine an effective dose of vinblastine which would inhibit elongation but not be lethal to the cells. The results of these experiments are presented in Table IX. Vinblastine sulphate proved to have very damaging effects. Cells were very irregular, many had pyknotic nuclei and the organization of the regenerate was disturbed. With control and colchicine experiments 50% of the samples developed a discernible regenerate, with vinblastine only 10-20% of the samples had a regenerate that could be staged. With a dosage of 4.4×10^{-8} M one of the two observable regenerates appeared to have some elongation although the regenerate was very small. The other regenerate was stopped at Stage IV. Using higher concentrations of vinblastine (4.4×10^{-6} M and 4.4×10^{-7}M) there was no elongation and very few depigmented cells were present.

Due to its damaging effects few other experiments were performed with vinblastine,for the few that were a dose of 4.4×10^{-7} M was used to insure inhibition of elongation. Figure 17 illustrates a few vinblastine treated regenerates.

Table IX

Dose Response Data for Vinblastine

Treatment	Dose	No. Observed No. Attempted	Stage	Apparent Age/ Average Apparent Age	Actual Age	Retardation
Control	-	4/5	VII-X	18-25/20	32	11
Vinblastine	4.4×10^{-6} M	1/10	III	8-11/10	31	21
Vinblastine	4.4×10^{-7} M	1/10	III	8-11/10	31	21
Vinblastine	4.4×10^{-8} M	2/10	IV-VI	9-16/12	31	19

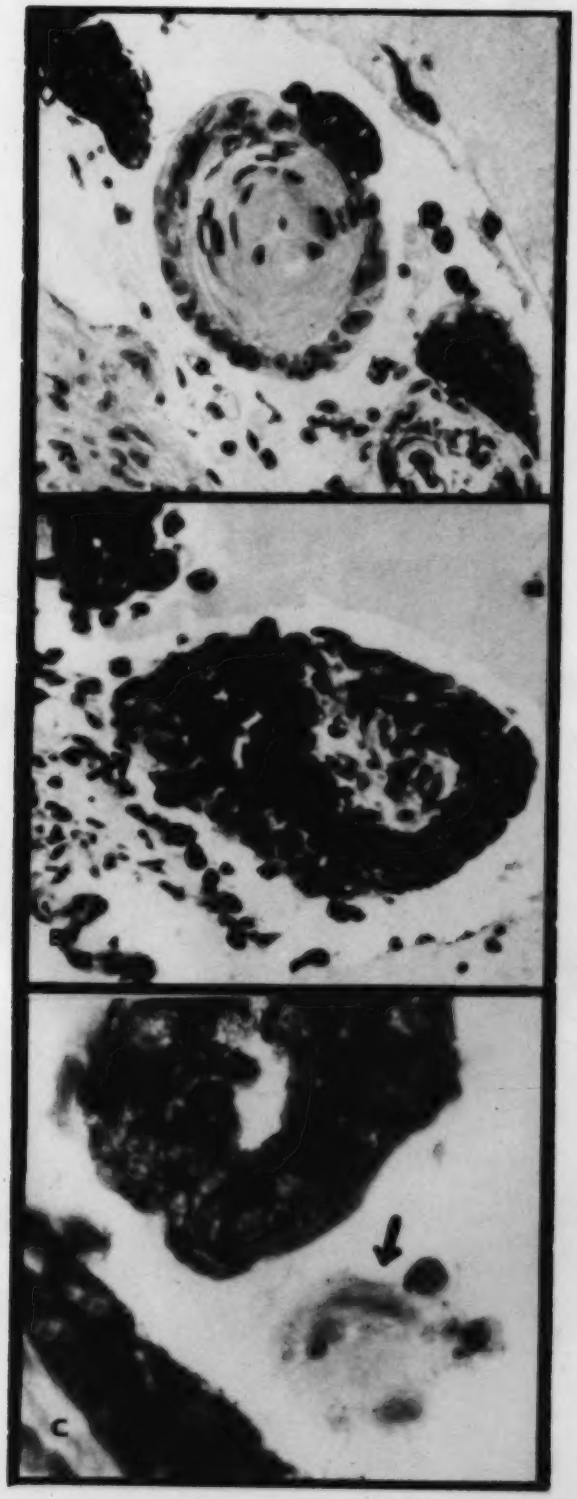
Figure 17

Micrographs of Control and Vinblastine Treated Regenerates Incubated for 1 Day *in vitro* and 15 Days *in vivo*

a - A Stage IX control treated iris. x 125

b - A very early stage vinblastine treated regenerate. Very little depigmentation has taken place (arrow). x 125

c - A vinblastine treated iris. The regenerate (arrow) is very diffuse and it is questionable whether or not any elongation has taken place. x 250


c

(b) Most Sensitive Stage

These experiments were attempted in order to determine whether vinblastine was inhibitory to the same stages of regeneration as colchicine. As can be seen from the data in Table X the effects of the drug were so drastic that an estimate of the most sensitive stage could not be made. However the 6 day regenerate that was observed showed inhibition of depigmentation and elongation.

E. Effects Due to Cytochalasin B

(a) Most Sensitive Stage

These experiments were undertaken to see if cytochalasin B had any effect on cell elongation. Colchicine presumably affects the microtubule system and cytochalasin B supposedly affects microfilaments. Therefore it was important to find out if either system or both are involved in elongation. Results presented in Table XI indicate that cytochalasin B has no observable inhibitory effect on any stage of regeneration. Similarly, DMSO has no observable inhibitory effect. Figures 18 and 19 compare cytochalasin B, DMSO, colchicine, and control effects for 10 and 15 day regenerates. As can be seen in Figure 18 control, cytochalasin B, and DMSO treated irises all show elongation, organized lentoid formation and similar stages of regeneration. Cytochalasin B treated regenerates did not differ significantly from the controls.

Table X

The Reaction of Various Age Regenerates to Vinblastine

Age of Iris Postlentectomy Days	Total Age Days	Stages of Control/Average Apparent Age	Stages of Vinblastine Average Apparent Age	Retardation	
				Control	Vinblastine
0	16	IV-VI/14	II-III/6	2	10
2	18	IV-VI/14	Degenerated	4	-
6	22	VI-VIII/17	III/19	5	13
10	26	VIII-X/18	Degenerated	8	-
15	31	VII-X/20	Degenerated	11	-

Table XI

The Reaction of Various Age Regenerates to DMSO and Cytochalasin B

Age of Iris Postlentectomy Days	Total Age Days	Stages of Control/Average Apparent Age	Stages of Treated/ Average Apparent Age		Retardation		
			Cyto B	DMSO	Control	Cyto B	DMSO
0	16	IV-VI/14	IV-VI/14	IV-VI/14	2	2	2
2	18	IV-VI/14	IV-VI/14	IV-VI/14	4	4	4
6	22	VI-VIII/17	VI-VIII/17	VII-VIII/17	5	5	5
10	26	VII-X/18	VII-X/18	-	8	8	-
15	31	VII-X/20	IX-X/20	VIII-X/20	11	11	11

Figure 18

Micrographs of 15 day Regenerate Irises Treated for 1 Day *in vitro* and 15 Days *in vivo*

A - Stage IX DMSO treated iris. x 125

B - Stage IX cytochalasin B irises. x 125

C - Stage VIII cytochalasin B treated iris. x 125

All of these regenerates demonstrate cell elongation and advanced stages of regeneration.

A
B
C

Figure 19

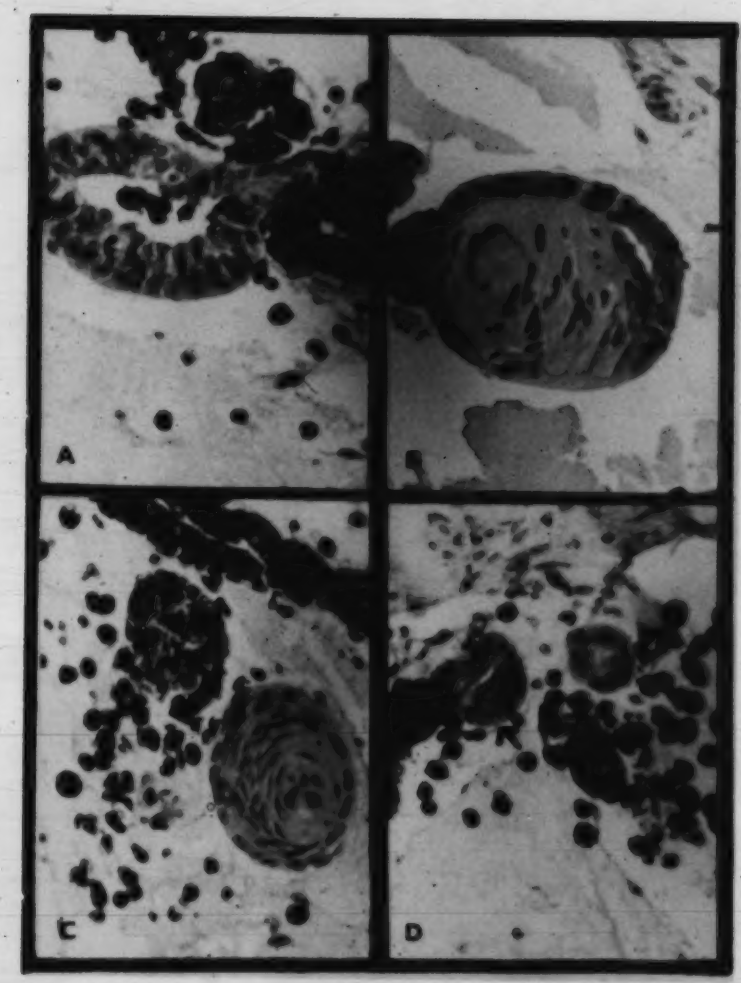
Micrographs of 10 Day Regenerate Irises Incubated for 1 Day *in vitro* and 15 Days *in vivo*

A - A Stage VII cytochalasin B treated iris. x 125

B - A Stage IX cytochalasin B treated iris. x 125

C - A Stage VIII control iris. x 125

D - A colchicine treated iris - Stage V - no elongation appears to be present. A lentoid from the host (arrow) shows elongation. x 125


A
B
C
D

2. Electron Microscopy

Rather than do a detailed electron microscope study of the different stages of regeneration, I concentrated on 15 day regenerates, the most colchicine sensitive stage. I confined my study to two types of cells; cells involved in the process of depigmentation and depigmented cells involved in elongation. The former also appear to be elongating because they are sloughing off cytoplasm at both ends of the cell.

A. Controls

Depigmented cells of 15 day regenerates were associated rather loosely in the lentoid. There were regions where the cells come close to one another; however, there seemed to be a scarcity of cell junctions. A few desmosomes were observed between some cells. The cells appeared to have many extensions or processes which intertwined.

The cytoplasm of these cells was rich in mitochondria, ribosomes, and profiles of rough endoplasmic reticulum. Microtubules and microfilaments were also apparent in most of the cells. Microtubules were for the most part paraxial to elongation; however, some were observed running perpendicular to the axis of elongation. There seemed to be three general areas that the microtubules were found in:

(a) in arrays just below the cell membrane (referred to as sub-surface microtubules), (b) fairly close to the nuclear membrane (referred to as perinuclear microtubules), and (c) in the intermediate cytoplasm (referred to as intermediate microtubules). It was difficult to ascertain any orientation for the microfilaments. See Figures 20, 21, and 22.

B. Colchicine

The organization and over-all appearance of the cells was the same as for the control treated cells. The cells were loosely associated and had many extensions. Colchicine treated cells were fixed immediately after treatment and in some cases 3 days after treatment. There did not appear to be any differences in ultrastructure between regenerates fixed at these times after treatment. There were no microtubules apparent in any areas of the cytoplasm although there were a few structures that looked like remnants of microtubules. Microfilaments were still visible in the cytoplasm. See Figures 23 and 24.

C. Cytochalasin B

Cells were similar to the controls in over-all appearance and organization . The cytoplasm of the cells contained microtubules in the same three areas as the controls. There did not appear to be any microfilaments present. See Figure 25.

Figure 20

Electronmicrographs of a section of Control Treated 15 Day Regenerate Irises

A - A low magnification view of a section from the lentoid region of a 15 day regenerate . Notice the loose relationship between the cells . Also note the extensions visible on some of the cells (arrows) . x 4,000

B - An area of an iris epithelial cell showing two of the three levels of microtubules - the subsurface array - smt , and the intermediate microtubules Imt . x 40,000

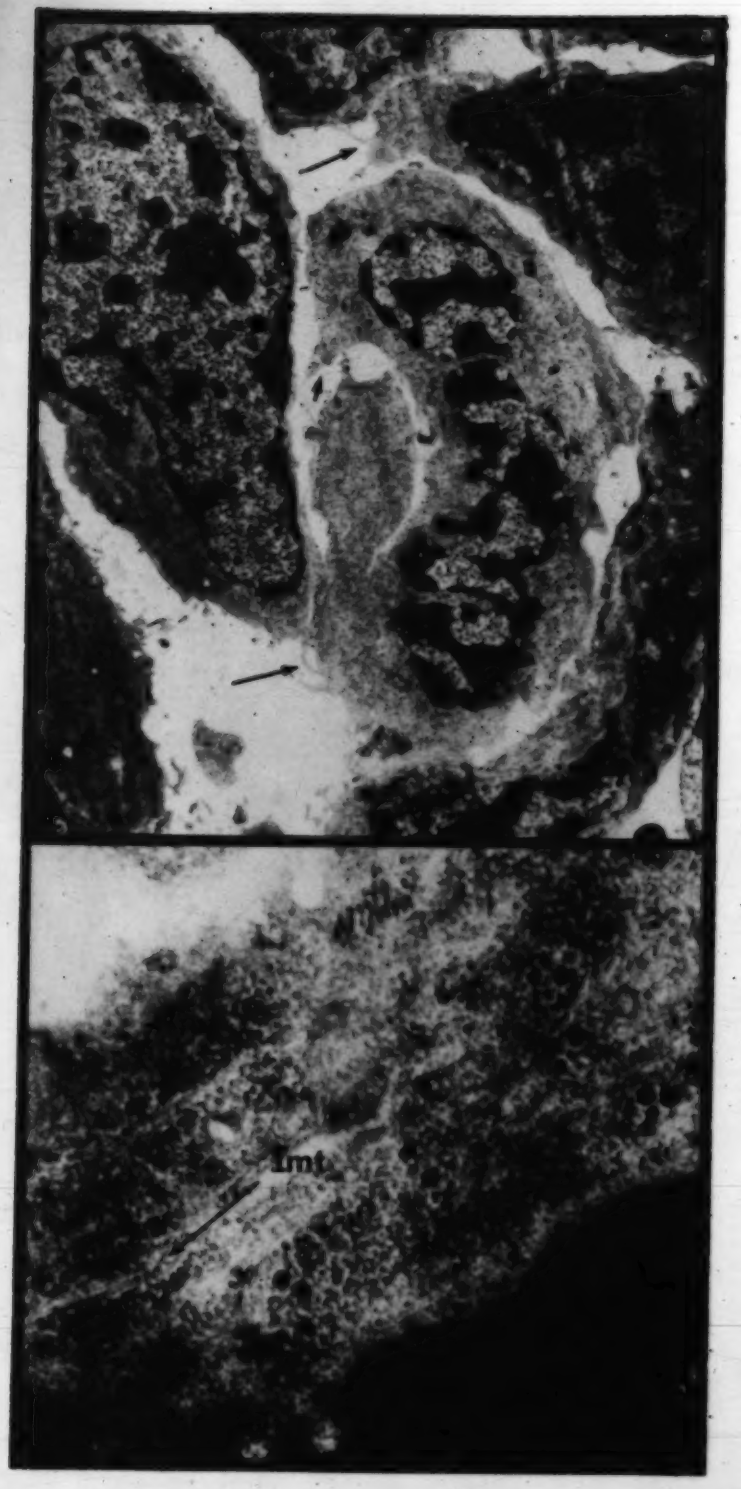

Imt

Figure 21

Electronmicrographs of Sections of Control Treated 15 Day Regenerate Irises

A- A section of a depigmented cell from the lentoid region . Sub-surface microtubules are visible in longitudinal and cross section - smt. Microfilaments are also visible just below the surface (F). x 40,000

B - A section of a depigmenting cell from a 15 day regenerate . Microtubules -(Mt) are visible running parallel to the long axis of the cell . x 14,000

C - An enlargement of (b) showing more details of the microtubules -(Mt) . x 30,000

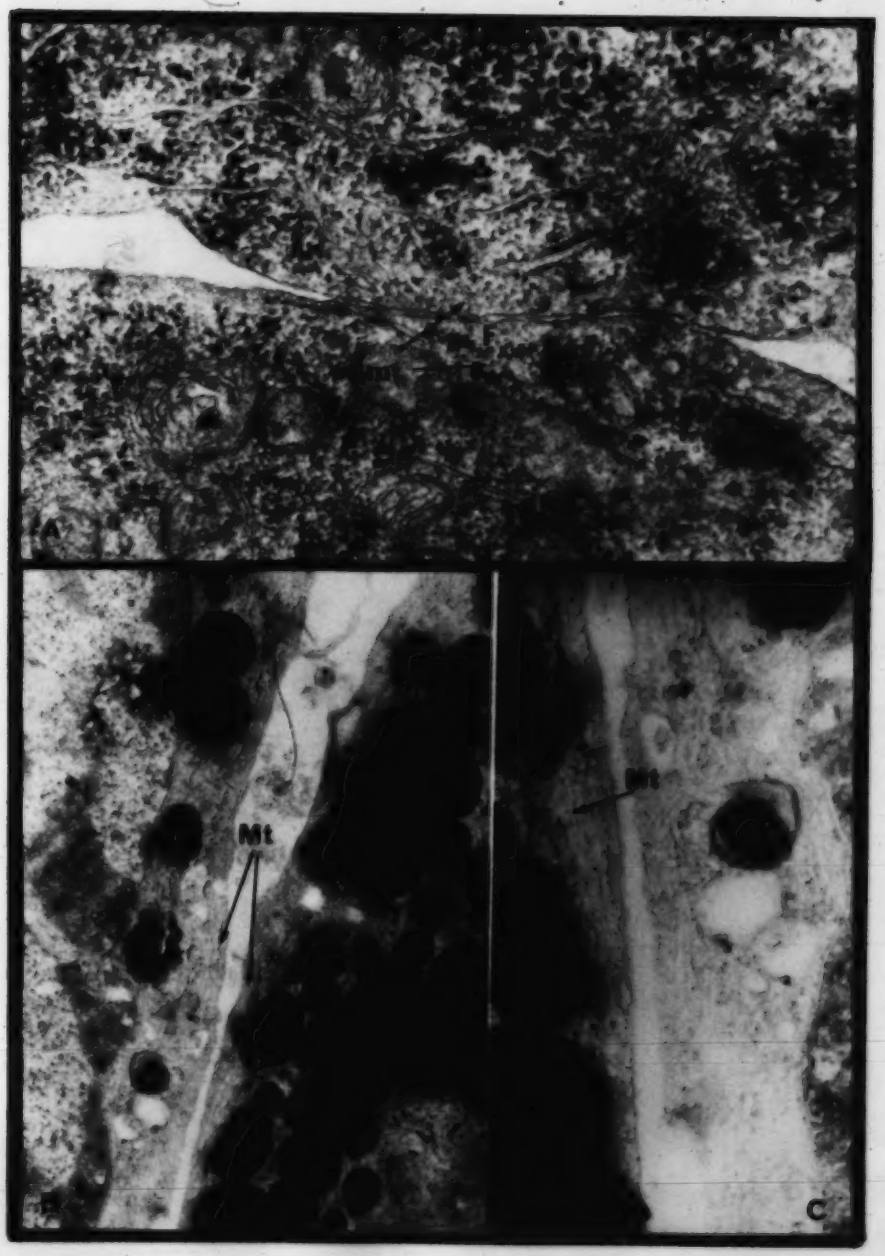

A
Mt
Mt
C

Figure 22.

Electronmicrographs of Sections of Control Treated 15 Day Regenerate Irises

A - A section of a cell from the lentoid region of a 15 day regenerate . Note the sub-surface microtubules - smt , and the desmosome (D) . x 55,000

B - A section of a depigmented cell from the lentoid region illustrating the deepest layers of microtubules running parallel to the nucleus - perinuclear microtubules - nmt . There are also cross-sections of microtubules visible in the intermediate and sub-surface regions . x 40,000

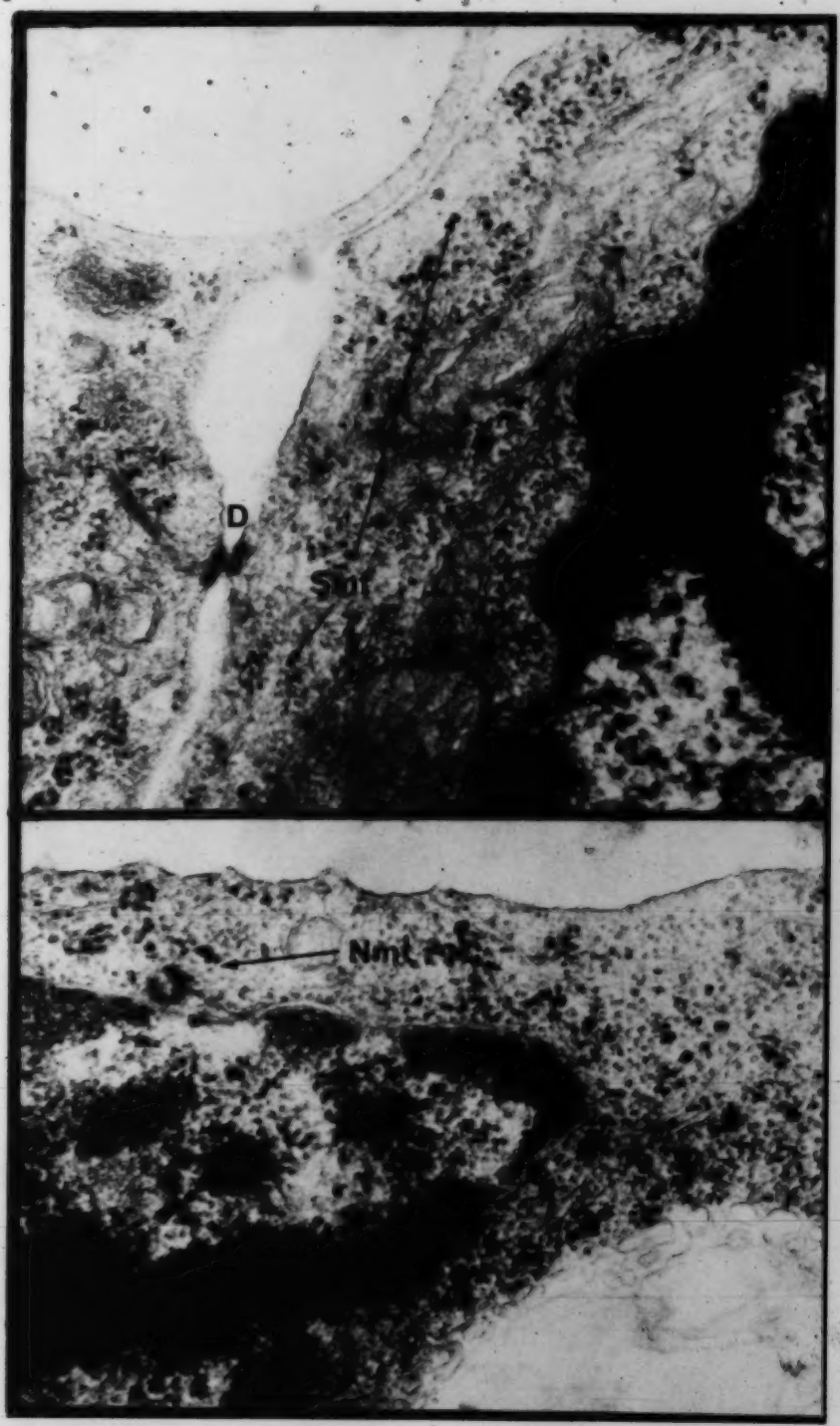

D
Sut
Nmt

Figure 23

Electronmicrographs of Sections of 15 Day Regenerate Irises Treated for 24 Hours With Colchicine

A and B - Notice the absence of microtubules from all three regions of the cytoplasm . Also note that cell extensions are still present (E) . x 40,000

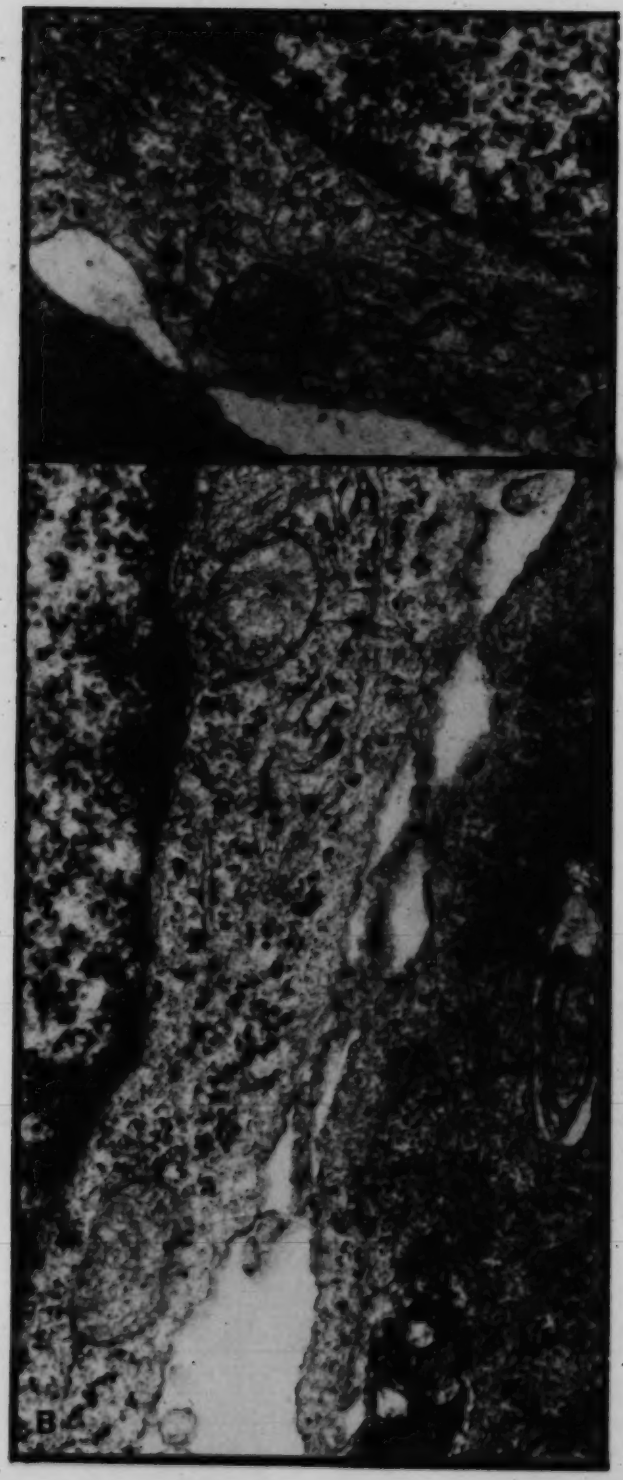

B

Figure 24

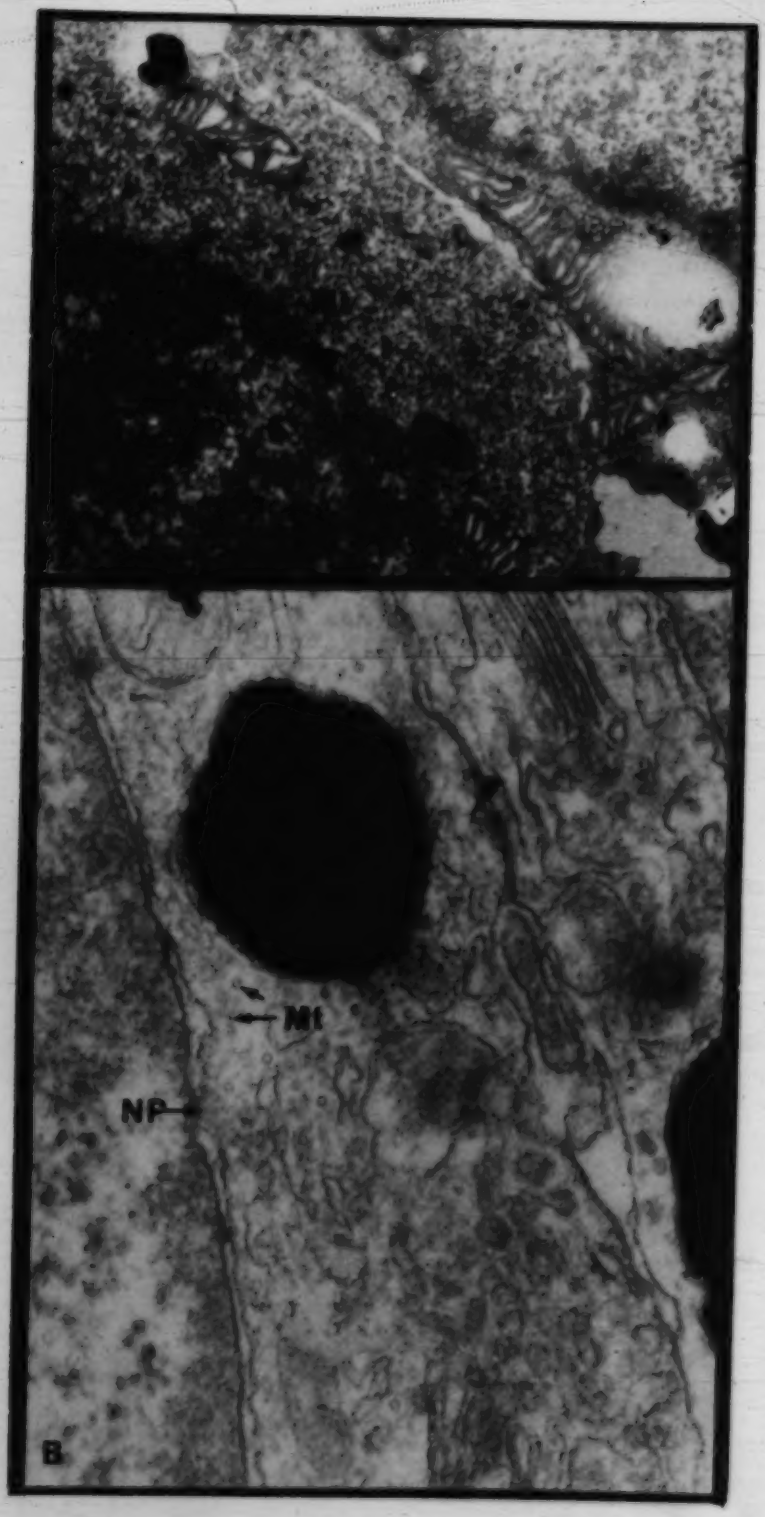
Electronmicrographs of Sections of 15 Day Regenerate Irises Treated for 24 Hours with Colchicine

A and B - Note the absence of microtubules in the various regions of the cytoplasm . In a few places there appear to be very short remnants of microtubules (R) . Microfilaments (F) are present . x 40,000

Figure 25

Electronmicrographs of Sections of 15 Day Regenerate Irises Treated for 24 Hours With 5 ug/ml Cytochalasin B

A and B - There do not appear to be any microfilaments present in these sections ; however, note that there are abundant microtubules in longitudinal and cross-section . A pore in the nuclear membrane is visible in B - Np .
A- x 7,000 , B - x 40,000


Mt
NP
B

D. Vinblastine

The purpose of this experiment was to determine if typical microtubule crystals could be identified in the cytoplasm of regenerates after treatment with vinblastine. Vinblastine has been used to precipitate microtubule protein *in vitro* and *in vivo* in several systems. (Bensch and Malawista 1968; Wilson *et al* 1970; and Bensch *et al* 1969).

In all of these systems it has been found that microtubule protein forms characteristic crystals under the influence of vinblastine. When 15 day regenerates treated with vinblastine were examined under the electronmicroscope many characteristic crystals were found in the cytoplasm of both depigmented and depigmenting cells. See Figure 26. Treated regenerates fixed 3 days after vinblastine treatment were also observed under the electronmicroscope. In these regenerates the crystalline structure appeared diffuse around the periphery and I interpreted this as evidence of the beginning of dissolution of the crystals.

Adult Newt Lens

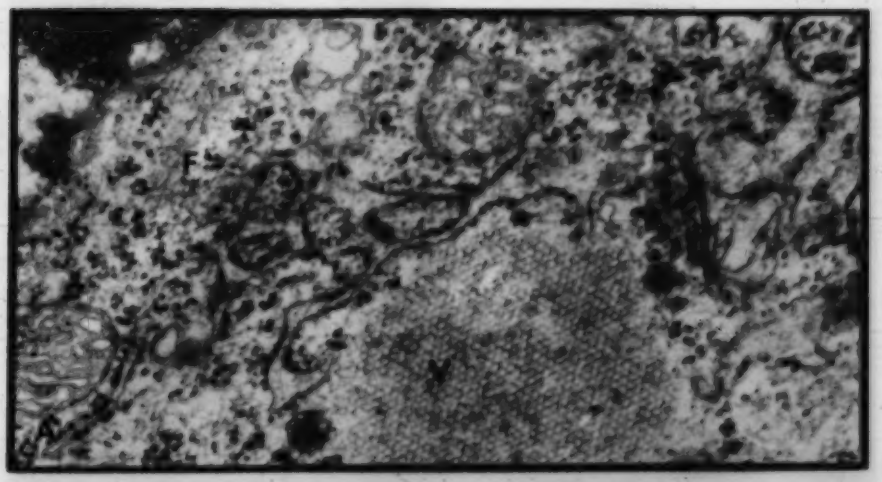
Adult lenses were very difficult to section; however, I was able to obtain a few sections. No intercellular space was evident between the cells. Many interdigitations were visible similar to those described by Kuwabara (1968) in human lens. Microtubules and microfilaments

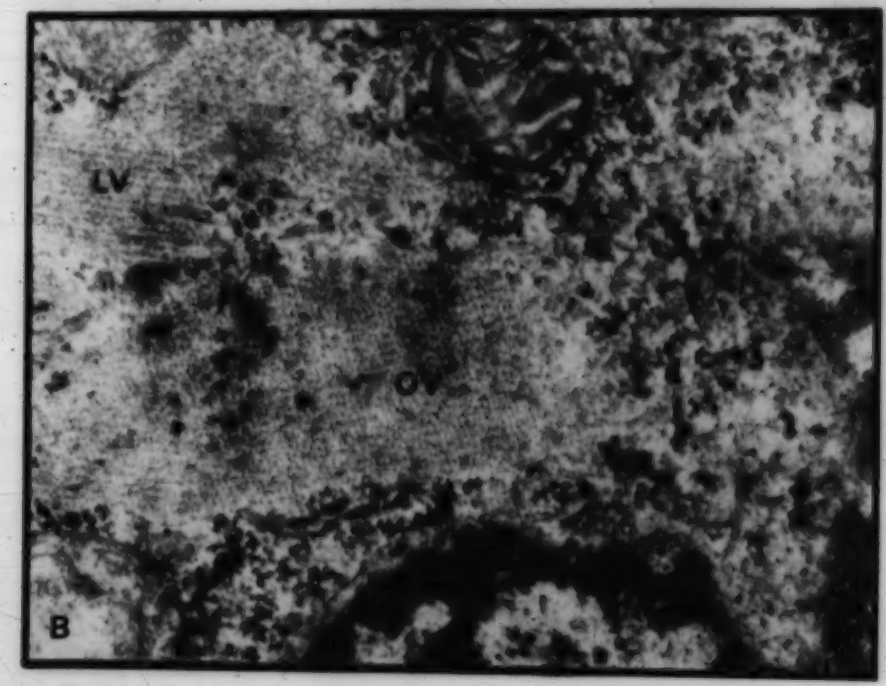
Figure 26

Electronmicrographs of Sections of 15 Day Regenerate Irises Treated with Vinblastine For 24 Hours

A- A typical vinblastine crystal in cross-section (V) is present . x 65,000

B - A vinblastine crystal sectioned partially in an oblique plane (OV) and in a longitudinal plane (LV). x 65,000


F
M
A


LV
O
B

were the only recognizable organelles in the cytoplasm. See Figure 27.

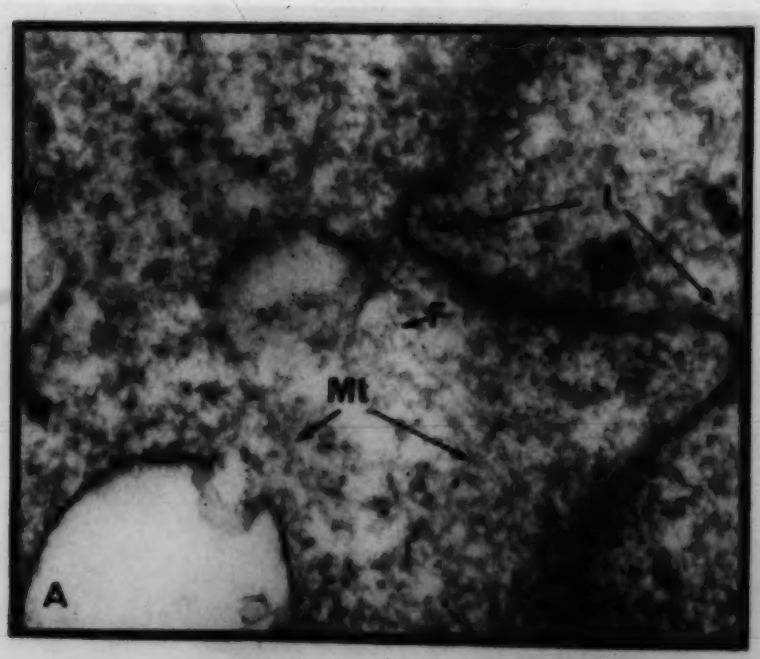
3. Cell Electrophoresis

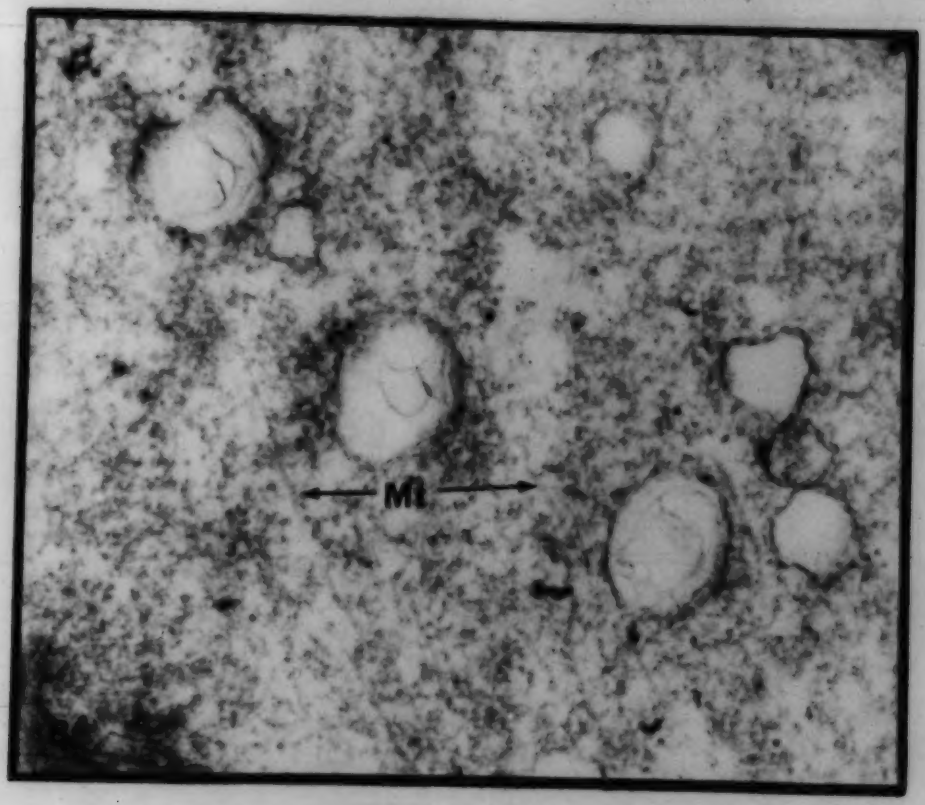
Colchicine has been shown to have an effect on cell surface mediated functions, presumably also microtubule dependent. Results from several investigators indicate that disruption of microtubules with colchicine or vinblastine has an effect on the distribution of certain specific sites on the cell surface (Ukena and Berlin 1972; Yahara and Edelman 1972, 1975). The general purpose of these experiments was to determine whether colchicine treatment affects the appearance of certain cell surface groups in the regenerating iris. It has been shown by Zalik and Scott (1972) that during regeneration various surface components which are sensitive to specific enzymes are present, these groups disappear during dedifferentiation and then reappear at the onset of redifferentiation. Neuraminidase sensitive groups that are present in normal iris and early stage regenerates, totally disappear from the cell surface by ten days after lentectomy when dedifferentiation is completed, and then reappear at or after 15 days after lentectomy. It was of interest to determine whether colchicine treatment would delay or alter the sequence of appearance of neuraminidase sensitive groups. Culture experiments already described have

Figure 27

Electronmicrographs of Sections of Adult Newt Lens

A and B - Both of these sections were taken from the outer region of the central core of the lens. Microtubules (Mt) and microfilaments (F) are present . Interdigitations (I) are very abundant between the cells . Note that the microtubules seem to run into the membrane of two adjacent cells . A x 40,000 , B x 20,000


Mt
A


Mt

shown that colchicine treatment of 15 day regenerate irises inhibits or slows down elongation and subsequent redifferentiation. It would be useful to see if colchicine treatment also slows down the apparent return to the differentiated condition which is evidenced by the re-acquisition of neuraminidase sensitive groups. The decrease in the electrophoretic mobilities of cells, induced by neuraminidase treatment, gives an indication of the presence of groups sensitive to this enzyme at the cell periphery.

A. Appearance of Neuraminidase Sensitivity

The first series of experiments were done to confirm the reappearance of neuraminidase sensitive groups. In addition, I wanted to determine if these groups would remain in the cells after 24 hours in culture. Table XII and Figure 28 show the results of these experiments. It appears that the neuraminidase effect starts to reappear in a 16 day (plus one day *in vivo* incubation) regenerate and that the response is definitely apparent in 17 day (plus one day *in vivo* incubation) regenerates cultured for 24 hours.

B. Length of Incubation Period in the Eye

It was estimated that a 14 or 15 day regenerate iris would be the best stage to test for colchicine sensitivity of membrane marker appearance since these are

Table XII

Appearance of Neuraminidase Sensitivity

* EPM Saline - electrophoretic mobility expressed in μ/sec/volt/cm of the cells from irises incubated for 24 hours in control medium. Dissociated cells were incubated with saline followed by EPM determination.

** EPM Neuraminidase - electrophoretic mobility expressed in μ/sec/volt/cm. Irises were incubated for 24 hours in control medium and dissociated cells were treated with neuraminidase followed by EPM determination.

*** Significance was determined by a paired T-Test. There was no significant difference where no figure appears in the table. A value of .01 or less was accepted as indicating significance.

(The actual age of the regenerate is the age appearing in the column- Age of Regenerate , plus 1 days for *in vivo* incubation.)

Table XII

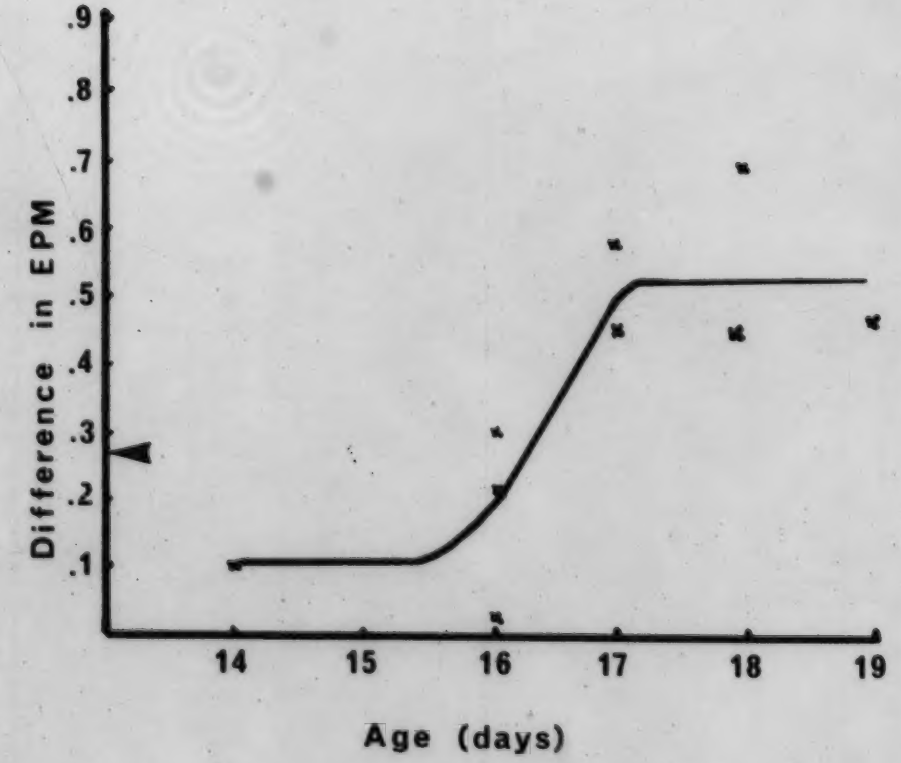
Appearance of Neuraminidase Sensitivity

Age of Regenerate (Days Postlentectomy)	EPM * Saline	EPM ** Neuraminidase	Difference	T-Test***
14	1.875	1.775	.100	-
15	-	-	-	-
16	1.904	1.73	.174	-
	1.951	1.919	.052	-
	1.821	1.40	.361	.005
17	1.94	1.48	.46	.005
	1.992	1.636	.356	.005
18	2.0	1.384	.616	.005
19	1.77	1.38	.39	.005

Figure 28

Graph of the Difference in EPM between Saline and Neuraminidase Treated Cells versus the Age of the Regenerate

The arrow indicates the usual difference in EPM that will give a significant value when the data are analyzed with a paired T-Test .


Difference in EPM
.9
.8
.7
.6
.5
.4
.3
.2
.1
14
15
16
17
18
19
Age (days)

the ages when surface markers are likely being produced. As mentioned previously these groups have appeared on the surface by 16-17 days postlentectomy. It was necessary to incubate the irises *in vivo* to allow the groups time to re-appear, therefore this experiment was undertaken to find out how long to incubate the irises *in vivo*. The results for this experiment are summarized in Table XIII. It appears from these experiments that colchicine affects the re-acquisition of neuraminidase sensitive groups in 14 day regenerates cultured for 4 days *in vivo*. Two days seem too short an interval--the neuraminidase effect is not always developed in regenerates at this age (total age 17 - a 14 day regenerate plus 24 hours *in vitro* incubation plus 2 days *in vivo*). Six and eight days are probably a rather long interval to incubate the iris, since it is possible that recovery from colchicine is already occurring and the neuraminidase effect starts to appear at the cell surface.

C. Sensitive Period of Regeneration

These experiments were undertaken in order to determine if there was a period of regeneration during which the re-appearance of cell markers (in this case neuraminidase sensitive groups) could be inhibited by colchicine treatment. Regenerates at 8 to 21 days after

Table XIII

Effect of Various *in vivo* Incubation Times on the Appearance of Neuraminidase Sensitive Groups on the Cell Surface

In Vivo Incubation Days	Control			Colchicine		
	Saline EPM	N'nase EPM	T-Test*	Saline EPM	EPM N'nase	T-Test
2	1.699	1.401	<.01	1.735	1.491	<.01
4	1.824	1.376	<.001	1.707	1.491	-
6	1.516	1.118	<.005	1.588	1.254	<.001
8	1.667	1.232	<.005	1.484	1.170	<.001

*T-Test--a value .01 or less indicates that there is a significant difference between the EPM of saline and neuraminidase treated cells.

lentectomy were cultured for one day in the presence or absence of colchicine and then were subsequently cultured *in vivo* for 4 days. Results (Table XIV) indicate that the majority of cells from 14 and 15 day regenerates treated with colchicine do not undergo a significant decrease in mobility after neuraminidase treatments while the mobility of the controls is significantly decreased by this enzyme. Figures 29 and 30 illustrate representative histograms from some of these experiments.

Results obtained with 8 and 10 day regenerates were unexpected because the total age of the irises at the time of estimation of electrophoretic mobility was 13 and 15 days respectively; ages at which the neuraminidase effect should not be present in controls, let alone in colchicine treated cells. The significance of this remains to be investigated. By 16 days control and colchicine treated regenerate cells possess neuraminidase sensitive groups at their surfaces, therefore there is no inhibition and colchicine can apparently not remove groups that have already appeared.

Only one experiment was performed using vinblastine in the incubation medium. It appears that a fourteen day regenerate is also subject to inhibition of the appearance of neuraminidase sensitive groups by vinblastine.

Table XIV. Sensitivity of Various Age Regenerates to the Inhibition of the Appearance of the Neuraminidase Effect by Colchicine.

*T-Test - a paired T-test was performed to test for significance. Where a line appears there was no significant difference. A value of .01 or lower indicated significance. Especially with the colchicine treated regenerates values of EPM's for neuraminidase and saline treated cells appeared to be significant but the T-test indicated that there was no significance. The arrow indicates one such case. In these cases the histograms appeared similar and did not show the spread evident in Figure 29.

Colchicine inhibited the neuraminidase response if there is a non-significant T-test in the colchicine column and a significant T-test in the control column.

Table XIV

Age of Iris Before Incubation Days Postlentectomy	Control Saline EPM	Control N'nase EPM	Control T-Test	Colchicine Saline EPM	Colchicine N'nase EPM	Colchicine T-Test
8	1.50	1.07	<.005	1.492	1.113	<.005
	1.553	1.256	<.01	1.526	1.262	<.01
10	1.9	1.3	<.005	2.05	1.01	<.005
13	1.49	1.34	-	1.76	1.55	-
14	1.824	1.376	<.005	1.69	1.707	-
	1.532	1.172	<.005	1.53	1.55	-
	1.622	1.178	<.005	1.649	1.621	-
	1.994	1.57	<.005	1.962	1.776	-
15	1.526	1.085	<.005	1.478	1.340	-
	1.806	1.343	<.005	1.776	1.534	-
	1.87	1.429	<.001	2.059	1.648	-
	1.556	1.236	<.001	1.546	1.452	-
16	1.549	1.25	<.001	1.68	1.20	<.001
21	1.65	1.12	<.005	1.592	1.22	<.001

Figure 29

Histograms of Mobility of Control Treated Iris Cells after Neuraminadase or Saline Treatment

Neuraminidase treatment tends to slow down cells that have neuraminidase sensitive groups on their surface , resulting in a histogram that is spread out .

EPM , electrophoretic mobility,is calculated by dividing a constant for the machine by the average time it takes a cell to travel 45 μ in an electrical field . The average is taken for twenty readings on a sample of cells .

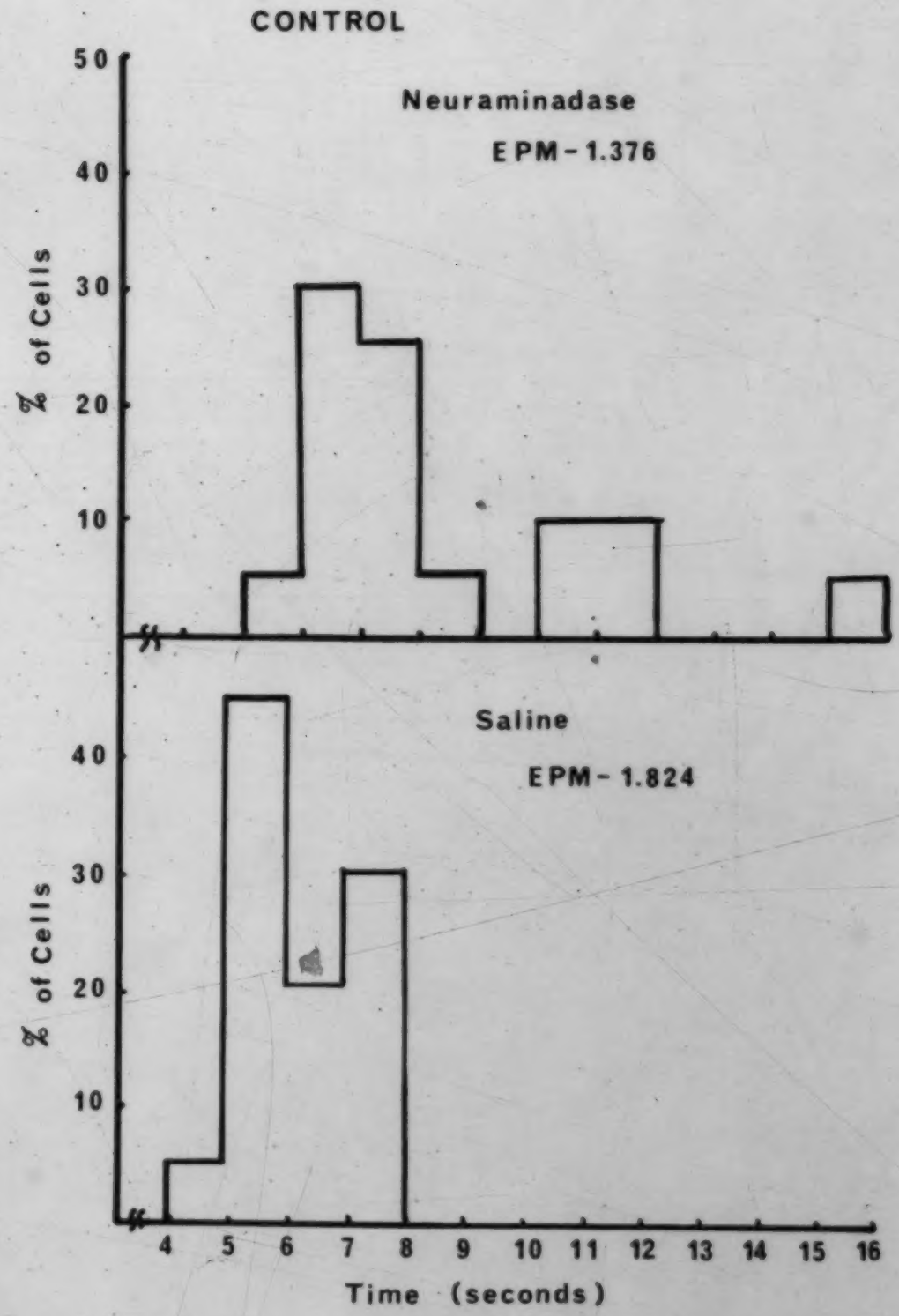
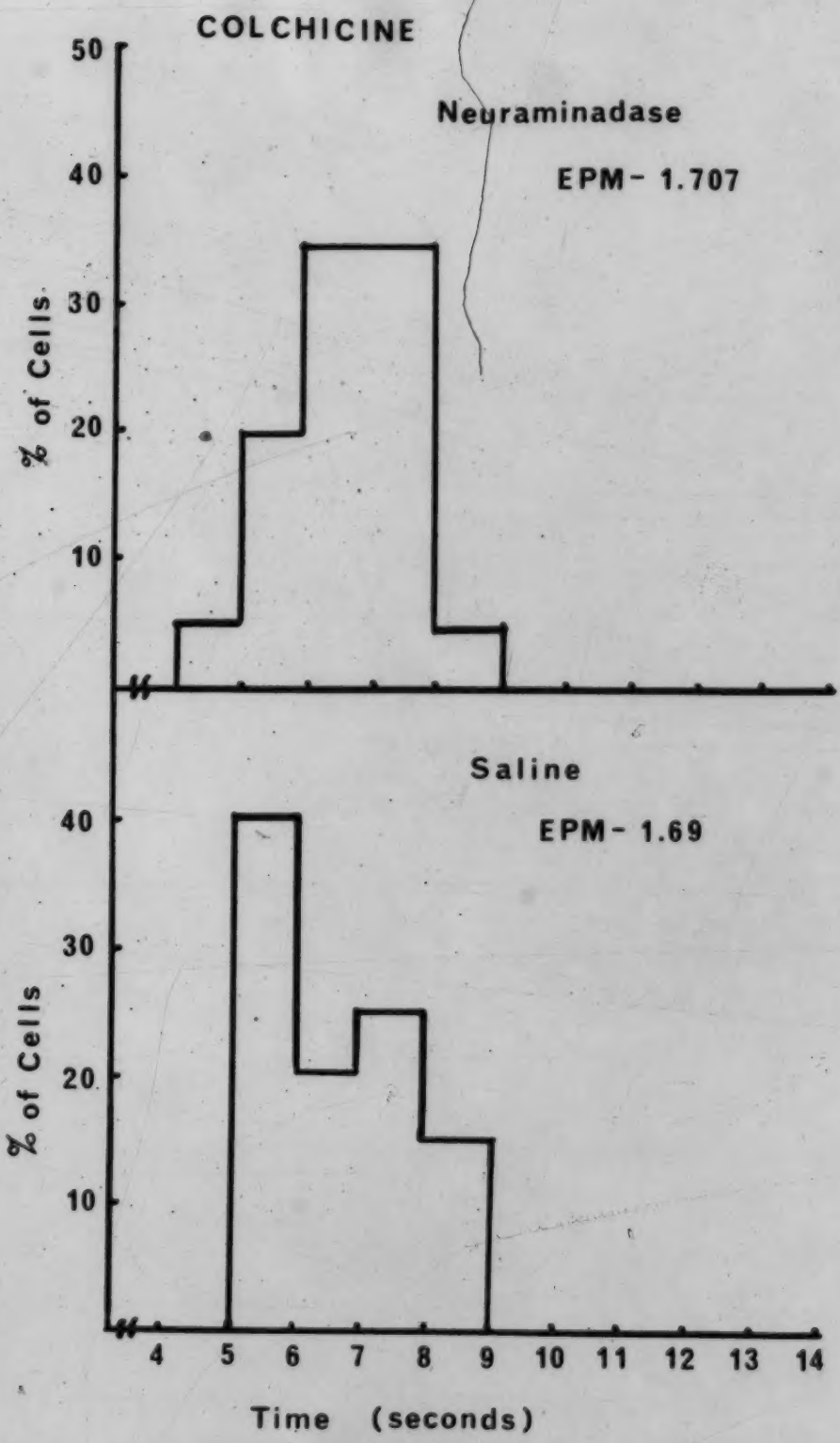

CONTROL
Neuraminadase
EPM - 1.376
% of Cells
50
40
30
20
10
Saline
EPM - 1.824
% of Cells
40
30
20
10
4 5 6 7 8 9 10 11 12 13 14 15 16
Time (seconds)

Figure 30

Histograms of mobility of Colchicine Treated Iris Cells after Neuraminidase or Saline Treatment

In this case colchicine inhibited the appearance of neuraminidase sensitive groups on the surface , therefore the histogram for neuraminidase treatment is not spread out .


COLCHICINE
Neuraminadase
EPM- 1.707
% of Cells
50
40
30
20
10
Saline
EPM- 1.69
% of Cells
40
30
20
10
4
5
6
7
8
9
10
11
12
13
14
Time (seconds)

DISCUSSION

A. Culture Conditions

Irises were removed from the eyes of previously lentectomized newts, were trimmed and separated from the cornea and then cultured for 24 hours in medium plus or minus various drugs. The irises were then washed in fresh medium and implanted in freshly lentectomized host newts for varying time periods. This method may seem like a rather complex way of treating a tissue with a drug; however, it has several advantages which I feel outweigh its disadvantages. First, it allows for a precise measure of the drug directly affecting the tissue being studied. Second, the drug only affects the tissue being studied and can not interfere with the metabolism of the whole animal. Third , the exposure time to the drug can be controlled. Fourth, *in vivo* conditions are maintained for the most part of the experiment. The main disadvantage of this method is that the iris is subject to some degree of mechanical damage from manipulations. This drawback can be overcome by estimating how much retardation is experienced by control treated irises and then considering that the drug treated irises would have the same amount of retardation due to experimental conditions as controls and that any further retardation was due to drug treatment. The retardation

experienced by the controls was referred to as intrinsic retardation, and as can be seen in the results, the older the regenerating iris was when manipulated, the greater the intrinsic retardation. This is to be expected, because as the newly forming regenerate develops and forms a lentoid it is very fragile and is more susceptible to mechanical damage. Since both the drug treated and control irises were handled in the same manner, it was assumed that they would experience similar levels of retardation due to manipulation.

The method used for determining the intrinsic retardation and drug retardation was rather unique and perhaps warrants further discussion. As previously mentioned several investigators have studied normal sequences of regeneration in different species of newts. This study uses Yamada's characterizations of Sato's stages. Specific stages of regeneration have been shown to occur at various intervals of time after lentectomy. The stages can be identified by certain criteria; such as degree of depigmentation, presence or absence of elongation and lens fiber cells, and the size and shape of the lentoid. By observing an iris that has been prepared for light microscopy and examining the above features it is possible to estimate what stage of regeneration it has reached. Once the stage is known, it is possible to estimate the time period at which this stage occurs.

Therefore, to calculate if any retardation was experienced by my regenerates; the treated irises were cultured *in vivo* for varying periods of time and then prepared for light microscopy and staged according to the criteria for normally developing regenerates. The usual age of the observed stage was assessed and compared with the actual age of the iris to see if any retardation had taken place. All treated irises were evaluated in this manner.

B. Colchicine

Colchicine has been known to man as a drug with miraculous powers for over 2,000 years. Ancient Egyptians, Greeks, and Hindus used *Colchicum* corms, seeds, and flowers for curing gout and rheumatism. Between 1938 - 1942 a great deal of literature was published on colchicine's ability to cause a "veritable explosion" of mitoses (Eigsti and Dustin 1955). Early workers on mitosis were able to observe that the continuous fibers of the mitotic apparatus were suppressed by colchicine (Peters 1946) but no one seemed to know how colchicine acted and how it arrested mitosis. Inoué (1952) by means of a study of birefringence in the spindle of dividing cells of *Chaetopterus pergamentaceous* , a marine annelid worm, proposed that colchicine likely functioned by "breaking down some chemical bond in or in between spindle micelles and simultaneously causing some of the remaining micelles to contract as well as further

breaking down the linkage between them." More recent work (Borisy and Taylor 1967a and b, and Weisenberg, et al 1968) has shown that the binding site of colchicine is situated on a protein which has a sedimentation constant of 6S. This protein is thought to be a microtubular sub-unit which is not only present in the mitotic apparatus but also in the cytoplasm of the cell in interphase. It is generally accepted that colchicine binds to microtubular sub-units and that generally "slow morphogenetic movements that involve microtubular polymerization tend to be colchicine sensitive." (Margulis 1973). Early workers thought colchicine had many non-specific side effects unrelated to mitotic arrest such as paralysis of the central nervous system, vomiting, and diarrhea ; however, present knowledge has shown that although these effects are often not due to mitotic arrest, they are due to a specific reaction of colchicine with microtubules. Colchicine is, however, still thought to have some effects not associated with microtubular function. Mizel and Wilson (1972) have shown that both colchicine and lumicolchicine (the latter a colchicine derivative with no anti-mitotic properties), are capable of inhibiting the uptake of nucleosides in mammalian cells. Nevertheless, colchicine is readily accepted as a specific inhibitor of functions involving microtubular assembly.

1. Dose Response

The effective doses found in these experiments, 2.5 x 10^{-5} - 1.25 x 10^{-3} M, generally agreed with doses used by other investigators in the field (Piatigorsky 1972a, Pearce and Zwann 1970, Karfunkel 1970). The dose used for the remainder of the experiments, 2.5 x 10^{-5} M did not show any apparent damaging effects on the cells, since regenerates proceeded further in depigmentation and were able to recover after appropriate time intervals of *in vivo* incubation.

2. *In Vivo* Incubation Time

Colchicine effects are immediately evident if the 15 day treated regenerates are fixed and observed with the electron microscope; no microtubules are evident in the cytoplasm. If irises are fixed and observed under the light microscope immediately after drug treatment there are no apparent differences between colchicine and control treated irises. However, if the irises are implanted in a lentectomized host and cultured *in vivo* it is possible to see that colchicine has an effect on subsequent elongation.

Experiments on the most effective *in vivo* incubation time indicate that 15 day irises incubated for 15days show the most retardation due to colchicine. Why? It is

possible that for shorter periods of incubation that the iris is still recovering from intrinsic retardation due to operational and culture conditions and colchicine retardation is not as apparent. Since a 15 day regenerate incubated for 5 days reaches stage V as does one incubated for 15 days, it might be assumed that the cells are blocked for at least 10 days at stage V; the stage where elongation is commencing. Colchicine regenerates incubated for 20 days *in vivo* begin to show evidence of some cell elongation. This argues against irreversible non-specific damage of this system by the conditions of drug treatment. My experiments suggest that there is a process occurring in a stage V regenerate that colchicine pre-treatment can block for at least 10 days. An *in vivo* incubation time of 15 days seems to allow the most differences between control and colchicine treated regenerates to develop .

3. Most Sensitive Stage

There are several processes involved in Wolffian lens regeneration that might be sensitive to colchicine inhibition: mitosis; depigmentation; and elongation. Mitosis is unquestionably sensitive to colchicine; however, is interference with mitosis great enough to account for the retardation observed in this system? Yamada and Roesel (1971) have shown that there are two peaks of mitosis during lens regeneration; one at 7 days

and one at 15 days postlentectomy. If inhibition of mitosis is responsible for most of the retardation experienced by regenerating irises, then there should be similar retardation times experienced by 7 and 15 day regenerates treated with colchicine . As can be seen in Figure 9, 15 day regenerates experience more retardation than do 7 day regenerates. Since there is an increase in retardation after 6 days postlentectomy it is possible that to a certain extent, some of this retardation may in part be due to inhibition of mitosis.

Pigment migration has been shown to involve microtubule integrity in other systems; *Fundulus* (Bikle *et al* 1966) and frog skin (Malawista 1965 and 1971). For these systems it was suggested that microtubules may define the channels and provide the motive force for pigment migration. It is quite possible that pigment migration with subsequent depigmentation, also involves intact microtubules in this system. Depigmentation usually commences at day 6 postlentectomy and continues through to day 9-15. Regenerates treated during these stages do not seem to show any inhibition of depigmentation since a large fraction of the cells making up the regenerate appear to be depigmented. Since there are more depigmented cells in a colchicine treated implant after 15 days *in vivo* culture than there were at the

time of treatment, it can be assumed that, at least, some depigmentation took place after colchicine treatment.

Several workers have suggested that microtubules are involved in elongation (Piatigorsky *et al* 1972a,b,c, Burnside 1971, and Arnold 1966). It is logical to consider that microtubules might be involved in cellular elongation during Wolffian lens regeneration also. The depigmented iris cells generally start to show signs of elongation at or after 15 days postlentectomy. Therefore it would be expected that regenerates treated with colchicine at 15 days postlentectomy would be retarded if microtubules are involved in cell elongation. Figure 9 not only shows retardation for treated 15 day regenerates, it also indicates that there is more retardation for irises treated at 15 days than for any other age treated. This alone would seem to indicate that microtubules are probably involved in important processes that occur at 15 days postlentectomy. Observation of the 15 day treated irises indicates that even after 15 days *in vivo* culture the cells still show no elongation. In addition to this, irises treated at earlier stages develop up to about a 15 day regenerate stage, arrested, and are then without any evidence of elongation. Thus irises treated with colchicine before or at the onset of elongation are unable to elongate and form lens fibers even after 15 days of *in vivo* incubation. Although

irises treated at 20 days postlentectomy still show retardation, they show some evidence of lens fiber formation. At this stage some cells would have already elongated while others would still be undergoing the process of elongation. The presence of some elongated cells in colchicine treated 20 day regenerates indicates that once elongation has taken place it can not be reversed by colchicine. These results agree with those reported by Pearce and Zwann (1970) in the embryonic chicken lens which showed that colcemid can not reverse elongation once it has occurred.

There is some degree of colchicine induced retardation experienced in regenerates at 5 days postlentectomy or after. Since there is a peak of mitosis at 7 days, much of the retardation of early stages may be due to mitotic arrest. However, the cells can not be retarded in mitosis for very long periods of time since in order to reach stage V a degree of mitosis and depigmentation should occur. In summary these results indicate that: 1) elongation is either appreciably retarded or inhibited by colchicine treatment; 2) a 15 day postlentectomy regenerate is most sensitive to colchicine treatment since it experiences the most retardation; and 3) it appears that once elongation has taken place it can not be reversed by colchicine treatment.

4. Autoradiography

The autoradiographic studies performed on 15 day regenerates indicated that colchicine treatment did not cause an appreciable inhibition of incorporation of ^{3}H-thymidine and ^{3}H-uridine. Inhibition of nucleoside uptake is one of the non-mitotic actions of colchicine in some systems (Mizel and Wilson 1972). Therefore colchicine effects on cell elongation were not due to this non-mitotic effect .

In this system the data on ^{3}H-leucine incorporation into protein are very interesting because they not only give an index of protein synthesis, but they also allow one to roughly determine when crystallin synthesis is beginning. Yamada and Takata (1963) have shown that the onset of crystallin synthesis , coincides with a sharp increase in incorporation of amino acid into protein, observable by autoradiography. A reasonable estimate of the onset of crystallin synthesis in my system can be made by observing the levels of ^{3}H-leucine incorporation at various times postlentectomy. Considering the data obtained from control cultured regenerates it appears that increased protein synthesis occurs first in a few cells of the regenerate, this agrees with the data of Takata *et al* (1964) who used fluorescent antibodies to detect appearance of lens specific protein. In my

system the increase in protein synthesis was first apparent at a total age of 21 days post-lentectomy. Yamada and Takata (1963) observed this increase between 9-15 days postlentectomy. Therefore it appears that my system is showing a retardation of between 6 and 11 days relative to the secondary enhancement of protein synthesis. These data are in agreement with the calculated intrinsic retardation of 8 days for a 10 day regenerate and 11 days for a 15 day regenerate. The data obtained on colchicine treated regenerates are quite different; although there is an increase in protein synthesis it is a more gradual increase and it never reaches the magnitude of the increase for the controls. The onset of the increase for colchicine treated regenerates is further delayed 5 days when compared with the controls. This delay is comparable to the 5 day delay calculated for colchicine treated regenerates using the staging method of comparing actual age with apparent age.

5. Immunofluorescence Studies

Regenerates 15 days after lentectomy, were incubated 15 days *in vivo* and then processed for fluorescent antibody staining. Controls showed well formed lentoids which fluoresced very brightly in all areas of the lentoid. There was fluorescence in the lens epithelium

although it was less intense than that in the fiber cell mass. The ^{3}H-leucine incorporation data indicate that there is a great increase in protein synthesis occurring. This is presumably associated with the synthesis of lens specific proteins. The bright fluorescence of the controls substantiates the idea that this increase in protein synthesis is due to the appearance of crystallins, the lens specific proteins. Examination of colchicine treated regenerates stained with fluorescent antibodies showed different results from the controls. Out of three regenerates studied, none showed any fluorescence comparable to the controls. One had a few very small bright regions of fluorescence which were localized in the interior of the depigmented cell mass. The other two showed no fluorescence. ^{3}H-leucine incorporation in colchicine treated regenerates indicated that there was a delayed increase in protein synthesis that never reached the magnitude of the control increase. The increase in ^{3}H-leucine incorporation had occurred by the age of the regenerates tested for immunofluorescence. There are two possibilities as to why this increase was not reflected in a production of lens specific proteins detectable by immunofluorescence: a) the level of the lens specific proteins was not high enough to be detected by the fluorescent antibody method,

although this method is usually very sensitive; b) it is possible that ^{3}H-leucine is incorporated into proteins which are non-lens specific. Piatigorsky *et al* (1972b) have shown that *de novo* synthesis of protein possibly microtubular protein is required for continued elongation in cultured chick lens epithelium. According to these results, cells which are unable to elongate are unable to synthesize detectable levels of lens specific proteins. These results are in agreement with those of Zwann (1975) who used mutant mice with lens defects. In his system lens fiber cells were unable to undergo elongation. He was able to show that in the absence of cell elongation the lens of these mutants will not produce any detectable levels of lens specific proteins. At the stage these regenerates were tested they were just beginning to overcome the block in elongation and it is possible that new microtubular protein had to be synthesized.

6. Lumicolchicine

Lumicolchicine treated regenerates appeared as control treated regenerates——there was no inhibition of cell elongation. This suggests that any effect that colchicine is having is due to its affect on microtubules since lumicolchicine mimics colchicine except in its antimitotic properties.

C. Other Drug Treatments

1. Vinblastine

Vinblastine was used to confirm the results obtained with colchicine since it is known to precipitate microtubules into characteristic crystals. The problem with vinblastine is that this drug is not as specific as colchicine and it functions differently. It precipitates microtubular protein into crystal lattices, whereas colchicine does not precipitate the microtubular protein; it binds with unbound monomer tubulin sub-units making it impossible for them to be polymerized as a microtubule. Moreover, vinblastine has also been reported to precipitate other cytoplasmic structural proteins (Wilson *et al* 1970) and ribosomes (Krishan and Hsu 1971). As mentioned, characteristic microtubular lattices were observed under the electron microscope in cells of 15 day regenerates treated with vinblastine. These results indicate that a sizeable population of microtubules is present in the cells of 15 day regenerates. However, cells after vinblastine treatment did not appear healthy, therefore experiments parallel to those with colchicine gave inconclusive results.

2. Cytochalasin B

Cytochalasin B has been reported to act by disrupting the microfilaments within the cytoplasm of cells ; however ,there has been a great deal of controversy over this hypothesized mechanism of action. Cohn *et al* (1972), Bluemink (1971), and Goldman (1972) have all suggested that cytochalasin B may act independently of a microfilament system. Cohn *et al* (1972) and Bluemink (1971) have both suggested that cytochalasin B is having its primary effect on the cell membrane. Whether its primary or secondary effect is on the microfilament system I think that most investigators would agree that microfilaments from the cytoplasm disappear after treatment with cytochalasin B. In the system under study cytochalasin B had no observable effects on elongation or regeneration regardless of the stage of regenerate. It is possible that microfilaments are not important in the processes that occur during regeneration in this system, but it is also possible that processes involving microfilaments have a minor or transitory role in development. Microfilament disruption may interfere with regeneration very briefly and would thus not be observable in this system. Microfilaments have been shown to reappear very rapidly after

cytochalasin B treatment has ceased, therefore it is possible that the microfilaments were not disrupted long enough for an observable effect to appear. Ortiz (unpublished results cited in Dumont and Yamada 1972) working on the same system *in vitro* found indications that cellular morphogenesis is suppressed by cytochalasin B.

D. Electron Microscopy

Observations with the electron microscope indicated that microtubules are present in the cytoplasm of the regenerating iris cells. They are generally oriented parallel to the axis of elongation. Colchicine treatment resulted in the disappearance of microtubules from the cytoplasm. Microtubules were absent from the cytoplasm for at least three days. The absence of microtubules could have contributed to the inhibition of elongation. Cells were very loosely associated in early stage lentoids where lens fiber cells were just beginning to form. In adult lens, the cells were very close together and had many interdigitations. This observation suggests that the formation of close attachments may be important in elongation.

E. Cell Electrophoresis

Recent work using lymphocytes has suggested that colchicine binding protein may be involved in the mobility

and distribution of lectin surface receptors on the cell membrane (Yahara and Edelman 1975, de Petris 1974, Oliver *et al* 1974). Most of these workers suggest that the colchicine binding protein is microtubular protein. De Petris (1974) has suggested that both microfilaments and microtubules may be involved in the internal cellular framework responsible for membrane movements. Wunderlich *et al* (1973) have interpreted their results on the effects of colchicine on membranes of *Tetrahymena pyriformis* differently from the interpretations of the above workers. They suggest that since colchicine is a lipophilic drug, it may dissolve in the apolar membrane regions and decrease the overall fluidity of the membrane by making membrane lipids more rigid. They based their interpretations on work with temperature induced changes observed in the freeze-fractured membranes.

Work by Zalik and Scott (1972) on regenerating irises has shown that various surface components which are sensitive to specific enzymes are present in the normal iris cell; these groups disappear during dedifferentiation and then reappear at the onset of redifferentation. The possibility arose, that these phenomena might also be inhibited by colchicine. Results using cell electrophoresis on irises cultured for 24 hours *in vitro* showed that in my system neuraminidase sensitivity has

reappeared by 17 days postlentectomy. I found that colchicine inhibited the appearance of neuraminidase sensitive groups only in 14 and 15 day regenerates cultured for 24 hours *in vitro* and then for 4 days *in vivo*. It can be seen from the results on incubation time in the eye that detection of this inhibition is very restricted in regards to the time of incubation *in vivo*. It appears that colchicine inhibition of the appearance of the neuraminidase sensitivity disappears if the iris is left much longer than 4 days *in vivo*.

It is probable that there is a colchicine sensitive process in a 14 or 15 day regenerate that is responsible for the organization or possible insertion 3 days later of surface groups that are sensitive to neuraminidase. As previously mentioned investigators working with lymphocytes have suggested that microtubules are possibly involved in membrane organization. The evidence, in my system, seems to indicate that a colchicine sensitive process, presumably involving microtubules, may be involved in the appearance of certain groups at the cell surface.

F. Mechanisms of Lens Fiber Elongation in the Lens Regenerating System

There are several suggestions as to the mechanisms involved in changes in cell shape in differentiating

systems. Most investigators tend to agree with the suggestions that both microtubules and microfilaments are involved to varying degrees in cell elongation. Piatigorsky (1972b) working with cultured chick epithelia, found that initial stages of lens fiber elongation involve utilization of a pool of microtubule sub-units, continued elongation required *de novo* synthesis of protein, possibly microtubule protein. He also found, however, that cytochalasin B could inhibit elongation of cultured lens epithelial cells. Other workers have suggested that microtubules and microfilaments co-operate to cause elongation (Yamada *et al* 1971) .

Some investigators suggest that microtubules are solely responsible for elongation. Burnside (1971) working in neural tube formation in the newt embryo suggested that microtubules may contribute to cell elongation by providing for a transport mechanism which would funnel cytoplasm components to the ends of the cell. They may also provide a cytoskeleton which would be involved in the stabilization of the asymmetrical cell shape. Two other possibilities were suggested. One possibility is the extension of microtubules by polymerization which would result in a "push" on the end of the cell. This mechanism would likely generate a force in a manner similar to that proposed by Inoué and Sato

(1967). These investigators suggested that chromosome movement is the result of the labile association of microtubules (depolymerization of microtubules shortens the spindle fiber resulting in chromosome movement toward the pole of the cell). The other possibility involves the sliding of microtubules over one another which would also generate a force on the poles of the cell. Microfilaments which are found in the system would supposedly be responsible for the apical constriction of the invaginating cells of the neural tube.

Some other mechanisms have been proposed to explain cellular elongation. Brown *et al* (1941) suggested that neurulation and accompanying cell elongation are a result of increased adhesiveness of cells of the presumptive neural tissue. More recently Zwann and Hendrix (1973) have suggested that cell and organ shape changes during early development of the occular lens are a result of; (a) tissue interaction, (b) differential cell division, and (c) cellular adhesion. In my system there is no limitation of area so that cell division could force cells to elongate because of limited space; however, I am interested in the idea that adhesion may be responsible for elongation. In my system, the cells that are beginning to elongate appear to have many extensions which intertwine with other cells. The cells are in very

loose contact with one another at this stage. Elongated cells in the adult newt lens are very closely apposed and have many interdigitations, therefore some mechanism has brought these cells closer together. It is possible that there is an increase in adhesion as cells elongate into fibers. This could result in contacts being established between the cells at points--possibly via the extensions. These localized contacts would increase in area so that the cell stretches out as it contacts cells parallel to it. Cellular organelles such as microtubules could channel the cells to stretch in one direction and thereby elongate.

Studies on sensitivity to colchicine inhibition of the appearance of neuraminidase sensitive groups at the cell surface showed that 14 and 15 day regenerates were sensitive to colchicine. Neuraminidase sensitive groups at the cell surface were the only ones tested for, and there is evidence to suggest that neuraminidase sensitive groups are involved in cell adhesion (Lloyd and Cook, 1974). It is quite possible that other cell surface groups which may or may not be involved in adhesion were also affected. Lackie (1974) has shown that colchicine can inhibit aggregation of polymorphonuclear leucocytes. In my system colchicine could have inhibited the appearance of surface groups affecting adhesion or it could have affected other groups which were already present at the cell surface.

Waddell *et al* (1974) have suggested 3 ways in which colchicine could affect adhesiveness: (a) by influencing the state of convolution of the cell surface; (b) by dispersing clusters of microtubule dependent adhesive sites; and (c) by causing a loss of polarity in the insertion of new membrane. Since 14 and 15 day regenerates are sensitive to colchicine inhibition of the appearance of neuraminidase sensitive groups on the surface and since 15 day regenerates are more sensitive to colchicine inhibition of elongation, it is possible that new surface groups essential to, or involved in cell elongation are appearing or are being organized at this stage. Electron microscope studies have shown that there is a sub-surface array of microtubules and these may be involved in the organization or insertion of specialized sites at the cell surface. Other microtubules deeper in the cytoplasm may contribute to elongation by some other means, such as: (a) forming a cytoskeleton which would support the change in cell shape; (b) directionally transporting materials ; or (c) providing a "push" at the ends of the cells.

Cytochalasin B had no noticeable effect in my system, so I have not included microfilaments in any of my theories of elongation as applied to the lens regenerating system. However, these organelles are present

and may be involved separately or in co-operation with microtubules in bringing about cell elongation .

SUMMARY

In summary, the main points made in this thesis are as follows :

1. Regenerates treated with colchicine before or at the onset of cell elongation are inhibited from beginning or furthering cell elongation.

2. Ultrastructural observations showed that microtubules are absent from cells of colchicine treated regenerates.

3. Lumicolchicine, a derivative of colchicine with no antimitotic properties, had no effect on cellular elongation.

4. The appearance of cell surface markers involves a colchicine sensitive process.

5. Cells in which cellular elongation is inhibited seem unable to synthesize lens specific proteins - the crystallins.

These results suggest that microtubules may have an important role in the elongation and differentiation of lens fibers in the lens regenerating system.

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